REMARKS

In the Office Action dated November 16, 2006, claims 1-18 and 79-137 are pending, of which claims 4-8, 17, 79-80, 89-99, 101, 107, 111-115, 121-123 and 126-137 are withdrawn from consideration. Claims 1-3, 9-16, 18, 81-88, 100, 102-106, 108-110, 116-120, and 124-125 are under examination and are rejected.

This Response addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claims 1-3, 9-16, 18, 81-88, 100, 102-106, 108-110, 116-120, 124 and 125 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

In Applicants' previous Response, independent claims were amended to delete the references to "derivative" and "equivalent" of GPI and to define the structure of the GPI molecule by its conserved core glycan. Dependent claims were also amended to delete those formulas that do not contain the conserved core glycan structure. The Examiner states in the instant Office Action that dependent claims 11, 86 and 116 still recite "derivative" and "equivalent" of GPI. Additionally, the Examiner indicates that dependent claims 11, 86 and 116 still contain formulas that do not contain the conserved core glycan.

Claims 11-13, 86-88 and 116-118 are also rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. The Examiner observes that claims 11, 86 and 116 depend from claims 1, 81 and 109, respectively, and recite the formulas "Mα2[Mα2][G]Mα6Mα4Gα6Ino-Y" and "Mα2[Mα2][X]Mα6Mα4Gα6Ino-Y", as well as "or derivatives or equivalents thereof". Base claims 1, 81 and 109 recite the limitation "wherein the

GPI molecule comprises Mα2Mα6Mα4Gα6Ino-Y". The Examiner contends that the formulas recited in claims 11, 86 and 116 do not comprise the core structure recited in the base claims.

By way of the instant amendment, Applicants have amended claims 11, 86 and 116 to delete the references to "derivative" and "equivalent" of GPI. Applicants further respectfully submit that all of the formulas presently recited in the claims, including those recited in claims 11, 86 and 116, contain the conserved core glycan structure. If the Examiner believes that a telephone interview or a diagram illustration is helpful, Applicants respectfully request that the Examiner telephone the undersigned attorney to clarify the issue.

In view of the foregoing, Applicants respectfully submit that the rejections under 35 U.S.C. §112, first paragraph (written description) and second paragraph, are overcome. Withdrawal of the rejections is respectfully requested.

Claims 1-3, 9-16, 18, 81-88, 100, 102-106, 108-110, 116-120, 124 and 125 are rejected under 35 U.S.C. §112, first paragraph, for lacking enablement.

In the first instance, the Examiner states that claims 11, 86 and 116 still recite "derivative" and "equivalent" of GPI. Applicants respectfully submit that this aspect of the rejection is moot in view of the deletion of these terms from claims 11, 86 and 116.

The Examiner also maintains that the specification does not provide enablement with respect to treatment or prophylaxis of *any* disease condition, in *any* mammal. Specifically, the Examiner alleges that the specification does not provide any working examples of treatment or prophylaxis of any condition or disease *in vivo*. Relying on Carvalho et al. and de Souza et al., the Examiner questions the applicability of GPI molecules to induction of clinical immunity, as well as the applicability of the observations made with a murine malaria model to humans.

Applicants previously submitted that this aspect of the enablement rejection should only apply, if at all, to claims 103, 109, 124 and their dependent claims, but not to claims 1, 18 and 81 or their dependent claims. The Examiner has not responded to Applicant's argument in this regard.

With respect to the Examiner's concern with respect to treating any and all diseases, Applicants wish to direct the Examiner's attention to the characterizations of the preferred disease conditions to be treated, as recited in the present claims. Specifically, claim 109 is directed to treating a mammalian disease condition characterized by a microorganism infection; and in dependent claim 120, such infection is a parasite infection. Claim 124 is directed to treating a mammalian disease condition characterized by the insufficiency or absence of an appropriate TH2 response; and in dependent claim 125, such disease is cerebral malaria, type I diabetes, autoimmune arthritis or systemic lupus erythromatosis.

The Examiner has cited de Souza et al. in stating that studies of the levels of anti-GPI antibodies from people living in areas of seasonal malaria transmission do not provide strong evidence that anti-GPI antibodies would confer resistance to malarial diseases. Applicants respectfully submit that many references contradict the position of de Souza et al., including Naik et al. (*J. Exp. Med.* 192: 1563, 2000) and Perraut et al. (*Microbes Infect.* 4: 682, 2005), attached hereto as **Exhibits 1-2**. Furthermore, Applicants respectfully submit that the de Souza et al. reference, relied upon by the Examiner, discusses the protective role of antibodies against GPI. This is irrelevant to the present invention, which is directed to the activation of T cells. T cells may provide protection where antibodies do not.

Applicants further respectfully submit that GPIs have been shown to activate NK-T cells (see, e.g., Schofield et al., *Science* 283: 225-229, 1999, attached hereto as **Exhibit 3**); and

these same T cells have been further demonstrated to protect again malaria (see, e.g., Hansen et al., *Immunity* 18: 391-402, 2003, attached hereto as **Exhibit 4**). Applicants respectfully submit that because these T cells are invariant and only recognize one dominant antigen (unlike conventional T cells, these T cells do not vary their T cell receptor repertoire), those skilled in the art would deduce, based on the present teaching, that expanding these T cells by exposure to GPI would provide protection against malaria. In this connection, Applicants direct the Examiner's attention to Figure 1 of the specification, which demonstrates that these T cells have a valuable effect in preventing disease in mice.

Moreover, in contrast to the Examiner's position, the *Plasmodium berghei* malaria infection of mice and rats is well recognized by a weight of scientific opinion to model the most important features of human malarial pathogenesis, and is used by many laboratories to provide a scientific basis and well accepted model for interventions against life threatening disease in humans. In this regard, Applicants provide the following scientific publications in support of the value of the murine malaria model: Schofield L. and Grau GE. (*Nat. Rev. Immunol. 5*: 722-735, 2005); Evans, K.J. et al. (*Blood* 107: 1192-1199, 2005); Barnell J. (*Blood* 1007: 854, 2006); and Lou et al. (*Clinical Microbiology Reviews* 14: 810, 2001), attached hereto as **Exhibits 5-8**. It is respectfully submitted that to those skilled in the art, an ability of GPI molecules to induce T-cell mediated immunity in *P. berghei*-infected mice, as shown in the present application, can be extrapolated to treating relevant diseases in other animals, including human.

In view of the foregoing, Applicants respectfully submit that the present specification provides sufficient guidance for those skilled in the art to practice the methods as presently claimed without undue experimentation. As such, Applicants respectfully submit that

the enablement rejection under 35 U.S.C. §112, first paragraph, is overcome. Withdrawal of the rejection is respectfully requested.

Claims 1-3, 9-16, 18, 81-88, 100 and 102 are rejected under 35 U.S.C. §102(b), as anticipated by Schofield et al. (*J. Exp. Med.* 177:145-153, 1993), as allegedly evidenced by a number of secondary references.

The Examiner contends that Schofield et al. (1993) teach administration of GPI, which would inherently result in GPI binding to CD1 and activation of Th cells, and therefore meet the limitation of the claims. Although the Examiner recognizes that Schofield et al. (1993) do not teach anywhere the structure of GPI molecules that is required to induce a CD1 restricted response, the Examiner contends, however, that the GPIs taught by Schofield et al. (1993) inherently have the core structure recited in the instant claims, as evidenced by the cited evidentiary references.

Applicants respectfully submit that the administration of the GPI molecules in the Schofield (1993) reference caused the death of the host by activating macrophages which produce TNF. This occurred due to the intraperitoneal administration of GPI together with D. galactosamine, causing hyperactivation of macrophages and, hence, death of the host. Further, in Schofield (1993), prior to administration of these molecules, the mice were subjected to intraperitoneal administration of thioglycollate in order to elicit a dominant macrophage population. This is a very specific form of bioassay, which was designed to promote the death endpoint due to the fact that the macrophages would preferentially interact with GPI's. This is an entirely different type of assay from the treatment method which is claimed and exemplified in the present application where the GPI's are designed to activate T cells to protect the host.

Accordingly, in light of the different treatment regime and the distinctly different endpoint, Applicants respectfully submit that Schofield et al. (1993) cannot be considered to have inherently disclosed the present invention. In particular, the fact that the mice were pretreated so as to skew them towards a macrophage response in the reference would in fact teach away from the inherent complexing of these GPI molecules to CD1 in order to induce NK1.1 T helper cell activation. Therefore, it is respectfully submitted that the §102(b) rejection based on Schofield et al. (1993) is overcome. Withdrawal of the rejection is respectfully requested.

Claims 11-13, 86-88 and 116-118 are rejected under 35 U.S.C. §102(a) as anticipated by Schofield et al. (*Science* 283:225-229, January 1999), as evidenced by van Joost et al. (*J. Amer. Acad. Dermatol.* 27:922-8, 1992) and Paul (*Fundamental Immunol.* 2nd Ed., 1989, New York, Raven Press, page 405).

In response, Applicants respectfully submit herewith a Katz Declaration **(Exhibit 9)**, signed by the present inventors, which is believed to have established that the inventors of the present application are the only inventors of the subject matter disclosed in Schofield et al. (1999). As stated in the Declaration, while there are five other individuals listed as co-authors on the Schofield et al. (1999) publication, they are not co-inventors of the subject matter described therein, and their contribution was limited to technical assistance.

Applicants respectfully submit that disclosure of a publication that occurred less than one year before Applicants' application comes within scope of §102(a) only if description is not of applicants' own work. In re Katz, 215 USPQ 14, 18 (CCPA 1982). Because the Declaration has sufficiently established that the work described in the Schofield et al. (1999) publication is not work by "another" within the meaning of §102(a), Applicants submit that the

Schofield et al. (1999) publication is not prior art under §102(a). As such, withdrawal of the §102(a) rejection based on Schofield et al. (1999) is respectfully requested.

As further set forth in the Office Action, the Examiner has rejected all pending claims under 35 U.S.C. §103(a) as allegedly obvious based on WO 99/52547, in combination with Gerold et al. (*J. Biol. Chem.* 269(4): 2597-2606, 1994) and Gerold et al. (*Mol. Biochem. Parasit.* 75: 131-143, 1996).

Specifically, the Examiner contends that WO 99/52547 teaches treatment of malaria or other parasitic infections by administering a CD1-binding GPI to induce a CD4+ T cell response. The Examiner admits that WO 99/52547 does not teach the treatment of malaria or other parasitic infections comprising a GPI that comprises the structure recited in the instant claims. However, the Examiner contends that the secondary references to Gerold et al. teach the structure of the MSP-1 and MSP2 GPIs from malaria. According to the Examiner, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have used the malaria GPIs taught by Gerold et al. as the GPI in the method taught by WO 99/52547.

In this regard, Applicants observe that the Examiner has withdrawn the previous anticipation rejection based on WO 99/52547. Applicants previously argued that even assuming, *pro arguendo*, that the GPI species recited in the present claims are not entitled to the priority date of AU PP 6758, these GPI species are not disclosed in WO 99/52547 either.

For the same reason, Applicants respectfully submit that WO 99/52547 cannot properly serve as a prior art reference to support the instant §103(a) rejection. In particular, WO 99/52547 has a publication date of October 21, 1999, i.e., after the priority date of the present application of October 27, 1998. The subject matter allegedly taught in WO 99/52547 and relied upon by the Examiner in raising the instant obviousness rejection, is present in

Applicants' priority document, AU PP 6758, filed before the publication of WO 99/52547. In particular, the priority document disclosed treatment of malaria or other parasitic infections by administering a CD1-binding GPI to induce a T cell response, preferably CD4 and NK1.1 T cell response. See pages 5-7 of the priority document AU PP 6758, for example.

Applicants further respectfully submit that once the alleged teaching of WO 99/52547 relied upon by the Examiner, is shown to be present in the Applicants' priority document, Applicants have effectively antedated WO 99/52547 and therefore have disqualified this document as prior art. It is well established that a reference is valid only for what it discloses, and if the applicant establishes priority with respect to such disclosure, the reference is of no effect at all. In re Stemple, 241 F2d 755, 760 (CCPA 1957). In other words, Applicants do not need to establish priority for every element in the claimed invention relative to this cited reference. All that Applicants are required to do is to antedate as much as what the reference discloses with respect to Applicants' claims.

Therefore, Applicants respectfully submit that WO 99/52547 does not constitute a proper "prior" art with respect to treatment of malaria or other parasitic infections by administering a CD1-binding GPI to induce a T cell response. Such deficiencies are by no means cured by the secondary references. Accordingly, it is respectfully submitted that the §103 rejection based on WO 99/52547 in view of the references to Gerold et al. is overcome. Withdrawal of the rejection is respectfully requested.

Claims 1-3, 9-16, 18, 81-88, 100, 102-106, 108-110, 116-120, 124 and 125 are also rejected under 35 U.S.C. 103(a) as obvious over WO 99/52547 in view of Schofield et al. (*Science* 283: 225-229, 1999). Further, Claims 1-3, 9-16, 18, 81-88, 100, 102-106, 108-110, 116-120, 124 and 125 are rejected under 25 U.S.C. 103(a) as obvious over WO 99/52547 (in

view of Schofield et al. (*J. Exp. Med.* 177: 145-153, 1993).

As submitted above, WO 99/52547 does not constitute a proper "prior" art with respect to treatment of malaria or other parasitic infections by administering a CD1-binding GPI to induce a T cell response; and the deficiencies are not cured by any of the secondary references. Accordingly, it is respectfully submitted that these §103 rejections based on WO 99/52547 are overcome. Withdrawal of these rejections is respectfully requested.

The Examiner has also rejected all pending claims under 35 U.S.C. §103(a) as obvious over WO 99/12562 A1 in view of various secondary references.

Essentially, the Examiner contends that WO 99/12562 A1 teaches treatment of parasitic infections in a mammal, including malaria, by administering a CD1-restricted antigen, such as a GPI. The Examiner admits that WO 99/12562 A1 does not teach the treatment of malaria or other parasitic infections comprising a GPI that comprises the structure recited in the instant claims. However, the Examiner contends that such a GPI molecule is taught by the secondary references.

Applicants respectfully submit that similar to WO 99/52547, WO 99/12562 A1 has a publication date of March 18, 1999, after the priority date of the present application of October 27, 1998. Applicants further respectfully submit that the subject matter allegedly taught in WO 99/12562, which is relied upon by the Examiner as the primary basis of the instant obviousness rejection, is present in Applicants' priority document. Specifically, Applicants' priority document discloses treatment of parasitic infections in a mammal, including malaria, by administering a CD1-restricted antigen, such as a GPI.

Therefore, with respect to treatment of malaria or other parasitic infections by administering a CD1-binding GPI, as presently claimed, Applicants have established priority

relative to WO 99/12562. WO99/12562 therefore does not constitute a proper "prior" art with respect to such claimed subject matter under In re Stemple, Id. Additionally, the deficiencies of WO99/12562 are not cured by any of the secondary references. Accordingly, it is respectfully submitted that all the §103 rejections based on WO99/12562 are overcome. Withdrawal of the rejections is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

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Glycosylphosphatidylinositol Anchors of *Plasmodium falciparum*: Molecular Characterization and Naturally Elicited Antibody Response That May Provide Immunity to Malaria Pathogenesis

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Abstract

Induction of proinflammatory cytokine responses by glycosylphosphatidylinositols (GPIs) of intraerythrocytic *Plasmodium falciparum* is believed to contribute to malaria pathogenesis. In this study, we purified the GPIs of *P. falciparum* to homogeneity and determined their structures by biochemical degradations and mass spectrometry. The parasite GPIs differ from those of the host in that they contain palmitic (major) and myristic (minor) acids at C-2 of inositol, predominantly C18:0 and C18:1 at *sn*-1 and *sn*-2, respectively, and do not contain additional phosphoethanolamine substitution in their core glycan structures. The purified parasite GPIs can induce tumor necrosis factor α release from macrophages. We also report a new finding that adults who have resistance to clinical malaria contain high levels of persistent anti-GPI antibodies, whereas susceptible children lack or have low levels of short-lived antibody response. Individuals who were not exposed to the malaria parasite completely lack anti-GPI antibodies. Absence of a persistent anti-GPI antibody response correlated with malaria-specific anemia and fever, suggesting that anti-GPI antibodies provide protection against clinical malaria. The antibodies are mainly directed against the acylated phosphoinositol portion of GPIs. These results are likely to be valuable in studies aimed at the evaluation of chemically defined structures for toxicity versus immunogenicity with implications for the development of GPI-based therapies or vaccines.

Key words: malaria parasite • cytokine response • antigenicity • acquired immunity • pathogenesis

Introduction

The rapid spread of drug-resistant *Plasmodium falciparum* and the lack of an effective malaria vaccine present an urgent need for alternative approaches to prevent malaria infection and pathogenesis (1). It is becoming increasingly clear that complete protection against malaria infection and illness requires multifactorial immunity (2). Currently,

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most vaccine efforts are aimed at immunity against infection (antiparasitic) by targeting the parasite proteins (2–5). However, it has long been suggested that immunity against severe malaria is partly antiparasitic and partly antitoxic (toxic effects in response to parasite factors). The majority of the adults in malaria endemic areas have resistance to severe malaria. However, most children <4 yr of age are susceptible despite exposure to high malaria transmission, which can produce high levels of antibodies against protein antigens including merozoite surface protein (MSP)[1]-1, erythrocyte membrane antigen (EBA)-175, and apical membrane antigen 1 (AMA)-1. Although antibody responses against parasite proteins correlate with protection against parasitemia (Branch, O.H., unpublished results), resistance to malaria illness is independent of parasitemia levels. This agrees with the resistance of adults and older children to malaria pathology even though they can develop significant parasitemia (6); conversely, severe illness can occur at relatively low-density parasitemias independent of antibody response against parasite proteins (7–9). The factors associated with the resistance to clinical disease (antidisease immunity) have not been established; understanding these would lead to alternative approaches for malaria control. In this regard, parasite glycosylphosphatidylinositols (GPIs) appear to offer new opportunities.

GPIs are a distinct class of glycolipids found ubiquitously in eukaryotic cells and implicated in several biological responses (10–12). GPIs are particularly abundant in parasites, where they are found as free lipids and attached to proteins. In intraerythrocytic *P. falciparum*, GPIs represent the major glycoconjugates. Several functionally important parasite proteins, including MSP-1, MSP-2, and MSP-4, are anchored to the cell membranes through GPI moieties (13–17). Recently, we have shown that *P. falciparum* synthesizes GPIs in a developmental stage–specific manner and that GPI biosynthesis is crucial for the development and survival of the parasite (18). The enzyme specificity of some key steps of parasite GPI biosynthesis differs significantly from those of the host, suggesting the possibility of targeting the parasite GPI structures for the development of antiparasitic drugs. However, detailed structures of parasite GPIs have not been determined. Although the structures of glycan cores have been established using metabolically labeled GPIs (19, 20), details regarding the nature of various acyl residues and other possible substitutents were not clear (21). Determination of a detailed structure requires isolation of pure GPIs which, in the case of *P. falciparum*, is a challenge due to the difficulty in obtaining adequate amounts of parasites free of host cell components. In this study, we were able to successfully purify *P. falciparum* GPIs to homogeneity and establish their structures.

[1]*Abbreviations used in this paper:* AHM, 2,5-anhydromannitol; CL, cardiolipin; EBA, erythrocyte membrane antigen; GalN, galactosamine; GC-MS, gas chromatography–mass spectrometry; GlcN, glucosamine; GPI, glycosylphosphatidylinositol; HF, hydrofluoric acid; HPTLC, high performance thin-layer chromatography; HRP, horseradish peroxidase; MSP, merozoite surface protein; PG, phosphoglyceride; PI, phosphatidylinositol.

It has long been believed that malaria pathology is due to factors endogenously produced in response to parasite toxins. Several studies have shown that malaria pathology is at least in part due to parasite toxic factors that can induce TNF-α and other cytokines, which could then lead to clinical effects including fever, hypoglycemia, dyserythropoiesis, and vascular damage in the lungs and brain (22, 23). This agrees with the elevated levels of TNF-α in patients with lethal cerebral malaria (24) and the ability of anti–TNF-α antibodies to prevent lethal cerebral pathology in mice (25).

P. falciparum GPIs have been identified as malaria pathogenicity factors based on their ability to induce inflammatory cytokines in macrophages and endothelial cells and cause symptoms reminiscent of acute malaria infection in experimental animals (26–29). Schofield et al. (26) have shown that parasite fractions enriched with GPIs can induce TNF-α and IL-1 in macrophages; in mice, GPIs can cause transient pyrexia, hypoglycemia, lethal cachexia, and even death in D-galactosamine (GalN)-sensitized animals. Schofield et al. have also shown that *P. falciparum* GPIs exert toxic effects through the expression of TNF-α, IL-1, inducible nitric oxide synthase (iNOS), and endothelial cell adhesion molecules by activating nuclear factor κB transcription factors (27–29). As *P. falciparum*–infected erythrocytes are sequestered in specific organs, the local high concentrations of toxic responses to the parasite GPIs can affect vital physiologic functions and cause severe illness. Recent studies have shown that GPIs from a *Trypanosoma cruzi* mucin can also induce proinflammatory cytokines (30). The antagonists of GPI-mediated signaling and a monoclonal antibody against *P. falciparum* GPIs can block the induction of toxic responses (27–29), suggesting that GPI-based therapy is possible.

Because *P. falciparum* GPIs are pathogenicity factors, we hypothesized that adults in malaria endemic areas should have GPI-specific protective immunity. We tested this hypothesis by analyzing the anti-GPI antibody response in sera from a longitudinal cohort study and in sera of a large group of adults from Western Kenya. The data demonstrate for the first time that people living in malaria endemic areas elicit a parasite GPI–specific IgG response in an age-dependent manner; although adults and older children have high levels of antibodies, malaria-susceptible children either lack or have only very low levels of short-lived antibodies. The results also suggest the involvement of anti-GPI antibodies in protection against malaria pathogenesis.

Materials and Methods

Reagents. Human blood and serum were purchased from Interstate Blood Bank. RPMI 1640, DME, and cell culture reagents were from Life Technologies. Gelatin, bee venom phospholipase A_2 (1,800 U/mg), standard phospholipids, and saponin were from Sigma-Aldrich. Silica Gel 60 high performance thin-layer chromatography (HPTLC) plates were from either EM Science or Whatman. Pronase was purchased from Calbiochem. *Aspergillus saitoi* α-mannosidase (400 mU/mg) and jack bean α-mannosidase

(30 U/mg) were from Oxford Glycosystems. Poly(isobutyl methacrylate) was procured from Polysciences, Inc. Horseradish peroxidase (HRP)-conjugated goat anti–human IgG (H and L chains) and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) reagent were from Kirkegaard & Perry Laboratories. Microtiter plates were from Dynex Technologies. [6-^{3}H]glucosamine (GlcN; 23 Ci/mmol), and [^{3}H]arachidonic (202 Ci/mmol), [^{3}H]myristic (55 Ci/mmol), and [^{3}H]oleic acids (8 Ci/mmol) were from Amersham Pharmacia Biotech. [^{3}H]Linoleic (60 Ci/mmol), [^{3}H]palmitic (60 Ci/mmol), [^{3}H]palmitoleic (60 Ci/mmol), and [^{3}H]stearic acids (60 Ci/mmol) were from American Radiolabeled Chemicals. ^{125}I-labeled goat anti–human IgG (8.66 μCi/μg) and En^{3}Hance were from NEN Life Science Products. Murine macrophages (J774A.1) were from American Type Culture Collection. HPLC grade solvents were used throughout the study.

Cell Culture and Isolation of Parasites. Intraerythrocytic *P. falciparum* (FCR-3 strain) was cultured in RPMI 1640 using human O-type erythrocytes and 10% O-positive human serum at 3–4% hematocrit (18, 19). Cultures were routinely synchronized with 5% sorbitol (31) and tested for mycoplasma (32); only contamination-free cultures were used.

Cultures with 20–30% parasitemia were harvested at mid-schizont stage. Packed erythrocytes (20 ml) suspended in 180 ml of 0.65% gelatin in culture medium were incubated at 37°C for 30 min (33). The enriched infected erythrocytes (70–80% parasitemia) in the supernatant were recovered and lysed with 0.015% saponin in Trager's buffer (34). The suspension was passed through a 26-gauge needle to solubilize the erythrocyte debris and then centrifuged. The parasites were suspended in the above buffer, layered on 5% BSA, and centrifuged (35). The packed parasites (~4 ml) were washed with the buffer and stored at −80°C until used.

Metabolic Labeling of the GPIs. Parasites were radiolabeled in medium containing 5 mM glucose and [^{3}H]GlcN (50 μCi/ml; reference 19). Labeling with ^{3}H–fatty acids (50 μCi/ml) was performed in medium containing 2% serum and 20 mM glucose.

Isolation of GPIs. All procedures were carried out using acid-washed, siliconized glassware to prevent surface adsorption of GPIs, high quality solvents, and sterile water and buffers. Parasites (10 ml) were lyophilized and extracted three times with 50 ml of chloroform/methanol (2:1, vol/vol) to remove nonglycosylated lipids. The pellet was extracted five times with 50 ml of CMW (chloroform/methanol/water, 10:10:3 [vol/vol/vol]), dried, and then partitioned between water and water-saturated 1-butanol. The organic layer was dried and the residue was extracted with 80% aqueous 1-propanol. Finally, GPIs were purified by HPLC and HPTLC.

To isolate GPIs that are linked to parasite proteins, the delipidated parasite pellet was digested with pronase (50 U/ml) in 50 ml of 100 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.0, at 37°C for 24 h. The released GPIs (designated as amino acid–linked GPIs) were extracted with water-saturated 1-butanol, washed with water, dried, and purified by HPLC. Control erythrocyte membrane debris, saponin-lysate, and ghosts were similarly extracted and fractionated.

HPLC Purification of GPIs. The GPIs (~10 μg plus 400,000 cpm of [^{3}H]GlcN-labeled GPIs) were chromatographed on a C_4 reversed phase Supelcosil LC-304 HPLC column (4.6 × 250 mm, 5 μm particle size; Supelco) using a linear gradient of 20–60% aqueous 1-propanol containing 0.1% TFA over a period of 80 min and held for 30 min at a flow rate of 0.5 ml/min (36). Fractions (1.0 ml) were collected, and elution of GPIs was monitored by measuring radioactivity. Aliquots (0.5 μl) were also assayed by ELISA with Kenyan adult sera. Glycolipids extracted from control erythrocyte membrane debris, total lysate, and ghosts were similarly chromatographed, and fractions were analyzed for reactivity with Kenyan sera.

Purification and Analysis of GPIs by HPTLC. The HPLC-purified GPIs (5 μg) were applied onto 10 × 10–cm plates as continuous streaks. Parallel spots with [^{3}H]GlcN-labeled GPIs (50,000 cpm) were used for monitoring GPI bands by fluorography using En^{3}Hance (18, 19). The plates were developed with CMW (10:10:2.5, vol/vol/vol). GPIs from the plates were extracted with CMW (10:10:3, vol/vol/vol), dried, dissolved in water-saturated 1-butanol, and washed with water.

In separate experiments, HPTLC plates were scraped (0.5-cm-width fractions), and GPIs were extracted and analyzed by ELISA using Kenyan adult sera. Glycolipids from control erythrocyte membrane debris, total lysate, and ghosts were similarly analyzed.

Compositional Analysis. HPLC- and HPTLC-purified, free and amino acid–linked GPIs (4–5 μg each), and hydrofluoric acid (HF)-released inositol-acylated carbohydrate moiety (2–3 μg), and diacylglycerol moiety (1–2 μg) were treated with 8 M anhydrous methanolic KOH for 2 h at 120°C. After acidification with cold dilute HCl, the fatty acid methyl esters were extracted with chloroform. Phospholipase A_2–released fatty acids were esterified with methyl iodide in DMSO/solid NaOH. Gas chromatography–mass spectrometry (GC-MS) was performed on a ThermoQuest GCQ^{plus} ion trap electron ionization quadrupole mass spectrometer (Finnigan) using DB-5 capillary column (30 m × 0.25 mm interior diameter with 0.25-μm film; Alltech). The temperature was held at 75°C for 1 min, raised to 150°C at 1°C/min, and then to 325°C at 8°C/min. GC retention times and mass spectra were compared against standards.

The free and amino acid–linked GPIs (~2 μg each) were hydrolyzed with 2.5 M TFA at 100°C for 5 h, and the hydrolysates were analyzed for mannose by HPLC (37).

Mass Spectrometry. Matrix-assisted laser-desorption/ionization time of flight mass spectrometry analysis was performed using a Kratos analytical MALDI-4 mass spectrometer equipped with a nitrogen laser at 20-kV accelerating voltage (38). Spectra were acquired with a time-delayed extraction and were the average of 50 laser shots. The matrix was saturated with α-cyano-4-hydroxycinnamic acid in 50% ethanol. The mass accuracy was within 2 daltons.

TNF-α Induction by GPIs. Murine macrophages (1.5 × 10^6 cells/well in 96-well plates) in DMEM and 10% fetal bovine serum were stimulated with 0.03–0.5 μM HPTLC-purified, free and amino acid–linked GPIs (see Fig. 1 B, lanes 2 and 4, respectively). TNF-α in the culture supernatants was measured by a quantitative sandwich ELISA using the TNF-α estimation kit (R&D Systems). Experiments were performed in triplicate with three separate batches of GPI preparations.

IgG-specific ELISA. HPLC-purified, free GPIs dissolved in methanol were coated (0.25–32 ng/well) onto 96-well microtiter plates. After evaporation of methanol at 37°C, the wells were blocked with 0.5% casein in 50 mM Tris-buffered saline, pH 7.4 (TBS-casein), and incubated with serially diluted sera (1:100 to 1:64,000) in TBS-casein containing 0.05% Tween 20. Initially, randomly selected 20 Kenyan and 2 USA adult sera were assayed. The bound antibodies were measured by HRP-conjugated goat anti–human IgG and ABTS substrate. All other sera were analyzed at 1:200 or 1:400 dilution by coating GPIs at 0.5–2 ng/well.

Competitive Inhibition ELISA. Sera were diluted 1:200 in TBS-casein containing 0.05% Tween 20 and incubated at room

temperature for 30 min with various concentrations of phosphatidylinositols (PIs), phosphoglyceride (PG), cardiolipin (CL), or GPI, and then ELISA was performed as described above.

TLC Immunoblotting. The HPLC-purified, free and amino acid–linked GPIs (100 ng each) were chromatographed on HPTLC plates. The plates were soaked in 0.1% poly(isobutyl methacrylate) in hexane, dried, and incubated for 2 h at room temperature with TBS containing 1% BSA and then for 2 h in 1:100 diluted sera (39). The plates were washed with cold phosphate-buffered saline, incubated with ^{125}I-labeled goat anti-human IgG (5 μCi/ml) for 1 h, washed, dried, and exposed to x-ray film.

Nitrous Acid Treatment. The HPLC-purified, free GPIs (20 μg) suspended in 150 μl 0.2 M NaOAc, pH 3.8, were treated with 150 μl 1 M $NaNO_2$ at room temperature for 24 h (40). The released PI moieties were extracted with water-saturated 1-butanol. The glycan moieties were recovered by chromatography on a Bio-Gel P-4 column (1 × 90 cm) in 100 mM pyridine, 100 mM HOAc, pH 5.2. These were used to assess seroreactivity.

Treatment with HF. The HPLC-purified, free GPIs (2 μg plus 500,000 cpm of [^{3}H]GlcN-labeled GPIs) were treated with 50% aqueous HF (50 μl) in an ice bath for 48 h (40). The reaction mixture was neutralized with LiOH and extracted with water-saturated 1-butanol. The organic layer was washed with water, dried, and the carbohydrate moiety was purified by HPLC.

Treatment with Mannosidase. The [^{3}H]GlcN-labeled GPI glycan cores (10,000 cpm) were digested with jack bean α-mannosidase and the products were deionized with AG 50W-X16 (H^+) and lyophilized (19). The glycan cores (5,000 cpm) were digested with *A. saitoi* α-mannosidase (0.5 mU/ml), deionized, and lyophilized (19).

Treatment with Phospholipase A_2. HPLC-purified GPIs (2 μg) in 100 μl 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, were treated with bee venom phospholipase A_2 (2,400 U/ml) at 37°C for 18 h. The digest was extracted with water-saturated 1-butanol and purified by HPLC; the retention time of *lyso*-GPIs is 75.5 min.

Analysis of Cohort Sera for Anti-GPI Antibodies. Sera were obtained from subjects of a longitudinal malaria project performed in a holoendemic, rural region of Western Kenya within the context of the Asembo Bay Cohort Project (ABCP [8]). Studies done in the subjects' households show that from March to May the entomological inoculation rate (EIR) was ~30 infected bites per person per month, whereas during the drier months the EIR was ~4. Pregnant mothers were identified and, after written consent, they, their delivered infants, and older children living in the same household were enrolled. The mothers and children were followed every 2 wk for ~4 yr, and clinical parameters, axillary temperature, and illness were recorded. Blood samples were taken monthly and parasite and hemoglobin density were measured. More than 90% of detected parasitemias contained *P. falciparum* species.

Sera of 100 7–8-yr-old children and 50 nonpregnant adults, collected during February through August 1993, were analyzed at two time points, spaced 1–3 mo apart. For data analysis, we selected two time points for the young children, in the same manner as the older individuals, when they were ~0.5, 1.5, 2.5, and 3.5 yr old. The study was approved by the Centers for Disease Control and Prevention and Kenyan Institutional Review Boards.

Analysis of the Relationship between Serum Anti-GPI Antibodies and Resistance to Malaria Pathogenesis. Analysis for correlation of anti-GPI antibody response with either hemoglobin or temperature as the dependent variable was done with the general linear model, Generalized Estimating Equations (41). The variables considered in the analyses were (a) anti-GPI antibody responder category (positive, intermittent, or negative), (b) parasite density, $\log_{10}(n + 1/\mu l)$, (c) age, and (d) antimalaria drug treatment. We controlled for the repeated measures on each patient. The estimated effect of positive and negative antibody responder category are given relative to that of the intermittent antibody responder category.

Results

Isolation and Purification of GPIs from Intraerythrocytic P. falciparum. As contamination with glycolipids from host erythrocytes and mycoplasma is a major concern in isolating GPIs from intraerythrocytic *P. falciparum*, we used rigorous protocols. First, parasite cultures were routinely tested for mycoplasma, and parasite-infected erythrocytes were enriched from cultures with 25–30 to 70–80% parasitemia to minimize erythrocyte components. Second, the parasites were released from infected erythrocytes by mild saponin lysis, washed, and purified by density centrifugation to remove erythrocyte membrane debris; metabolically [^{3}H]GlcN-labeled cultures revealed that this procedure did not release parasite GPIs. These procedures eliminated all of the erythrocyte components. Third, the purified parasites were differentially extracted to remove most nonglycosylated lipids, and the extract containing the free GPIs (not linked to proteins) was subjected to solvent fractionation to eliminate soluble hemozoin and other colored components. Finally, the GPIs were purified by successive fractionation using HPLC and HPTLC. The GPIs that are linked to parasite proteins were isolated after exhaustive digestion of the delipidated parasites with pronase, and are referred to as amino acid–linked GPIs. The yields of the HPLC-purified free and amino acid–linked GPIs were 100–120 and 25–30 μg per 10 ml of packed purified parasites, respectively, based on the mannose content.

The parasite GPIs were purified by HPLC using a C_4 reversed phase column, which separated matured GPIs from GPI intermediates (Fig. 1 A). The elution of GPIs was monitored by radioactivity, which overlapped with the reactivity of the GPIs to human sera containing anti-GPI antibodies (Fig. 1 A). The compounds isolated from the extracts of erythrocyte debris, lysates, and ghosts were fractionated in parallel with the purification of parasite GPIs by HPLC (Fig. 1 A) and HPTLC and analyzed as controls. These did not react with Kenyan adult sera (Fig. 1 A), suggesting that the activity is specific to parasite GPIs.

The GPIs were further purified by HPTLC; they migrated as broad major bands and in some instances separated into two major bands (Fig. 1 B). The heterogeneity of GPIs is due to the variation in the fatty acid composition of individual GPIs (see below). The HPTLC-purified GPIs (Fig. 1 B, lanes 2 and 4) contained mannose and GlcN in molar ratios of ~4:1 and were susceptible to nitrous acid, HF, and alkali (not shown). Fatty acid compositional analysis of HPLC-purified GPIs showed the presence of myristic

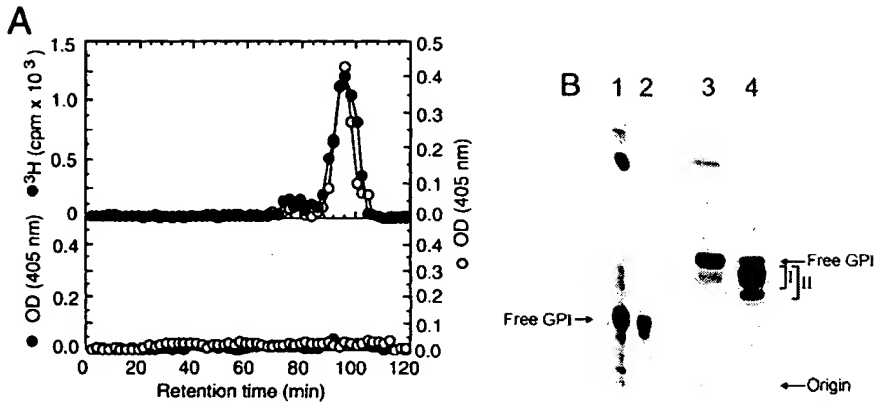


Figure 1. HPLC and HPTLC purification of *P. falciparum* GPIs. (A) The parasite GPIs (10 μg plus 400,000 cpm of [^{3}H]GlcN-labeled GPIs) were chromatographed on a 4.6 × 250–mm C_4 reversed phase HPLC column with a linear gradient of 20–60% aqueous 1-propanol (reference 37). (Top) Analysis of parasite GPIs: fractions (1.0 ml) were collected and ^{3}H activity in 5-μl aliquots was measured (●); 0.5-μl aliquots assayed by ELISA for immunoreactivity with Kenyan adult sera (○). (Bottom) Analysis of glycolipids from control erythrocyte membrane debris obtained from 4 ml packed erythrocytes (●), and those of delipidated, pronase-digested erythrocyte ghosts from 2 ml packed erythrocytes (○). 1-μl aliquots were assayed for immunoreactivity with Kenyan sera. Extracts of total erythrocyte lysate were similarly analyzed (not shown). (B) Fluorograms of the [^{3}H]GlcN-labeled GPIs chromatographed on Silica Gel 60 HPTLC plates using CMW (10:10:2.5, vol/vol/vol). Lane 1, total free GPIs before HPLC; lane 2, HPLC-purified matured free GPIs; lane 3, total free GPIs (different preparation from that in lane 1; obtained by culturing parasites in regular medium after replacing medium with radiolabeled precursor to maximally convert intermediates into matured GPIs); and lane 4, HPLC-purified, amino acid–linked GPIs. Each lane contains 200 ng of GPIs plus 20,000 cpm of [^{3}H]GlcN-labeled GPIs. Note that a small amount of free GPIs that remained with parasite pellet even after exhaustive extraction with organic solvents was copurified with amino acid–linked GPIs (lane 4).

(~4%), palmitic (~33%), stearic (~26%), oleic (~28%), cis-vaccenic (~2%), linoleic (~3%), arachidic (~2%), and behenic (~1%) acids, and 1–2% of unidentified acids. The presence of cis-vaccenic acid in the GPIs agrees with the ability of *P. falciparum* to synthesize two isomeric C18:1 acids (oleic and cis-vaccenic acids [42]).

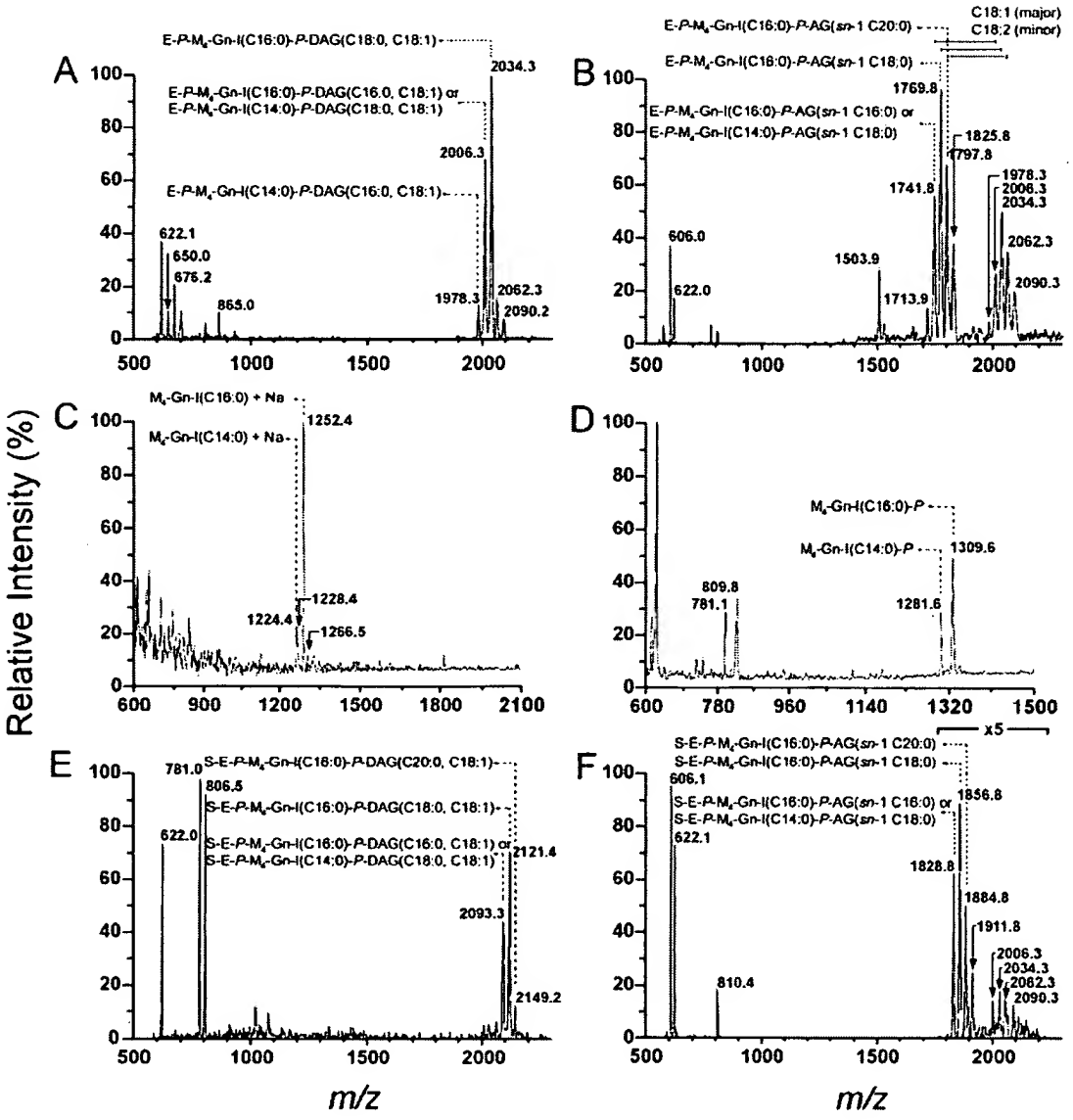
Structural Analysis of P. falciparum GPIs. The structures of the purified *P. falciparum* GPIs were determined by specific chemical and enzymatic degradation studies using [^{3}H]GlcN- and ^{3}H–fatty acid-labeled GPIs and by mass spectrometry.

The [^{3}H]GlcN-labeled GPIs were characterized by subjecting them to various degradative procedures. Treatment with jack bean α-mannosidase quantitatively converted the [^{3}H]GlcN-labeled GPIs into species with three mannose residues, similar to the results recently reported (18), suggesting that the distal fourth mannose residue does not contain any substituent. The [^{3}H]GlcN-labeled GPIs were dephosphorylated with HF and then deaminated and reduced with $NaBH_4$. The neutral glycan core, thus obtained, on HPTLC migrated as a single band with a mobility relative to the solvent front (R_f) value identical to previously characterized Man_4-2,5-anhydromannitol (AHM) (19). Treatment of the glycan core with *A. saitoi* α-mannosidase shifted the R_f value to that of authentic Man_2-AHM, and the product of jack bean α-mannosidase digestion comigrated with authentic AHM (not shown; 19). These results are consistent with the previously established structure of Manα1-2Manα1-2Manα1-6Manα1-4GlcN for the glycan core of *P. falciparum* GPIs (19, 20).

The mass spectrum of the purified, free GPIs (upper half portion of the HPTLC band in Fig. 1 B, lane 2) contained prominent molecular ions $(M-H)^-$ at *m/z* 2,006.3 and 2,034.3 and minor ions at *m/z* 1,978.3, 2,062.3, and 2,090.3 (Fig. 2 A). The observed difference of 28 mass units (in some fractions 26 units) between the consecutive molecular ions suggests that the GPIs contain mixtures of homologous fatty acids differing by two carbons. The proportions of the GPI species varied considerably depending on the regions of HPTLC band analyzed (compare Fig. 2, A and B), suggesting heterogeneity with respect to acyl substitutents. Mass spectrometry of the control materials did not show molecular ions comparable to the parasite GPIs (not shown). These results show that the purified compounds are GPIs of the parasite, not of erythrocytes.

Treatment of free GPIs with phospholipase A_2 resulted in the marked loss of $(M-H)^-$ ions of intact GPIs and the appearance of a new set of ions, each 264.5 mass units lower (Fig. 2 B), indicating that the GPIs contain a C18:1 acyl substituent at the *sn*-2 position. The spectra of some GPI fractions treated with phospholipase A_2 also contained significant levels of ions 262.5 mass units lower than the parent ions, suggesting the presence of GPIs with a C18:2 acid at *sn*-2. GC-MS analysis of phospholipase A_2–released fatty acids demonstrated the presence of predominantly oleic acid with minor amounts of cis-vaccenic and linoleic acids. These data together with the total fatty acid composition indicate that the parasite GPIs contain mainly oleic (~85%), cis-vaccenic (~6%), and linoleic (~9%) acids at *sn*-2.

As reported previously (21), the GPIs were sensitive to GPI-specific phospholipase D, but completely resistant to PI–phospholipase C (data not shown), suggesting the presence of an acyl substituent on C-2 of inositol. To identify this substituent, the GPIs were treated with HF and the released inositol-acylated carbohydrate moiety was purified by HPLC as a single symmetrical peak with a retention time of 47 min. Fatty acid compositional analysis indicated that the HF-released inositol-acylated carbohydrate moiety contained palmitic acid (~90%) and myritsic acid (~10%). Positive ion–mode mass spectrum showed $(M+Na^+)$ ions at *m/z* 1,224.4 and 1,252.7 (Fig. 2 C), which were assigned to Man_4-GlcN-inositol with myristoyl and palmitoyl substitution, respectively, on inositol. The signals at *m/z*



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Protein	GPI attachment site
MSP-1	GIFCS ↓ SSNFLGISFLLILMLILYSFI
MSP-2	SLLSN ↓ SSNIASINKFVVLISATLVLSFAIFI
MSP-4	LSLAS ↓ SSLNLIFNSFITIFVVILLIN

Figure 2. Mass spectrometry analysis of *P. falciparum* GPIs. HPLC- and HPTLC-purified GPIs were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry in negative or positive ion mode. (A) Negative ion mass spectrum of the purified free GPIs (Fig. 1 B, lane 2). (B) Negative ion mass spectrum of phospholipase A_2–treated free GPIs showing $(M-H)^-$ ions of unconverted GPIs and GPIs lacking substituent at *sn*-2 position. (C) Positive ion mass spectrum of inositol-acylated glycan moiety released by HF treatment of GPIs. (D) Negative ion mass spectrum of inositol-acylated glycan moiety released by HF treatment of GPIs. (E) Negative ion mode mass spectrum of the amino acid–linked GPI fraction I (Fig. 1 B, lane 4). The mass spectrum of fraction II (Fig. 1 B, lane 4) contained $(M-H)^-$ ions at *m/z* 2147.3 at significantly higher proportions compared with fraction I, in addition to an ion at *m/z* 2175.3 (not shown). (F) Negative ion mass spectrum of phospholipase A_2–treated amino acid–linked GPIs showing $(M-H)^-$ ions of unconverted GPIs and GPIs lacking substituent at *sn*-2 position. (A–F) The major GPI(s) that represent(s) the molecular ions are indicated. E, ethanolamine; P, phosphate; M_4, Man_4; Gn, GlcN; I, inositol; AG, acylglycerol; DAG, diacylglycerol; S, serine. (G) The cleavage sites for the attachment of GPIs in *P. falciparum* MSPs.

1,228.7 and 1,266.8 represent $(M+H^+)$ and $(M+K^+)$ ions of the carbohydrate with palmitoyl substitution. Of several mass spectra recorded, some (not shown) gave a reasonable signal at *m/z* 1,200.4, which was assigned to the $(M+H^+)$ ion of the carbohydrate with myristoyl substitution. A significant portion of the HF-released glycan moieties retained a phosphate ester group as indicated by the negative ion mode spectrum, which showed molecular ions at *m/z* 1,280.0 and 1,308.1 (Fig. 2 D). The phosphate ester group that survived HF treatment is likely the one linked to inositol because the adjacent protonated GlcN residue will render the hydrolysis of the phosphate ester bond to be kinetically slower. These results established that the inositol residue is substituted with palmitate (~90%) and myristate

(~10%). The diacylglycerol moiety released on treatment of GPI with HF contained predominantly C18:0 and C18:1 (two isomers), minor proportions of C14:0, C16:0, C18:2, C20:0, and C22:0, and unidentified acids. As C18:1 and C18:2 are present at *sn*-2 (see above), the remainders of the above acids should be located at *sn*-1.

The mass spectra of the HPTLC-purified, amino acid–linked GPIs (fraction I in Fig. 1 B, lane 4) containing one amino acid after pronase digestion showed prominent molecular ions that are 87 mass units higher than those of free GPIs (compare Fig. 2, E and F, with A), indicating that these GPIs contain a Ser residue. GPIs with attached Asn were also evident from $(M-H)^-$ ions at *m/z* 2120.3, 2148.3, and 2176.3 in the mass spectrum (not shown) of fraction II (Fig. 1 B, lane 4). This agrees with the predicted amino acids in GPI-anchored surface proteins (13–15), MSP-1, MSP-2, and MSP-4 (Fig. 2 G). Phospholipase A_2 treatment caused reduction by 264.5 mass units in the *m/z* of each of the molecular ions (Fig. 2 F), confirming the presence of predominantly C18:1 acyl residues at *sn*-2.

The acyl substituents in the GPIs were also studied by metabolic labeling with [^{3}H]myristic, [^{3}H]palmitic, [^{3}H]palmitoleic, [^{3}H]stearic, [^{3}H]oleic, [^{3}H]linoleic, and [^{3}H]arachidonic acids. All except arachidonate were incorporated into the parasite GPIs (Fig. 3 and data not shown). Treatment of the GPIs labeled with [^{3}H]myristic and [^{3}H]palmitic acids with phospholipase A_2 showed the presence of incorporated label only in the *lyso*-GPIs; the released fatty acids were not radioactive (Fig. 3). Upon treatment with HF, the radioactivity was found to be present on both the diacylglycerol and inositol carbohydrate moieties (not shown). In the case of [^{3}H]stearic acid, the radioactivity was incorporated only to the diacylglycerol moiety (not shown). These results agree with the presence of saturated fatty acids at *sn*-1 and at C-2 of inositol, but not at *sn*-2, established by mass spectrometry. The data also suggest that the parasite does not incorporate saturated fatty acids as unsaturated acids to the GPIs they synthesize or vice versa.

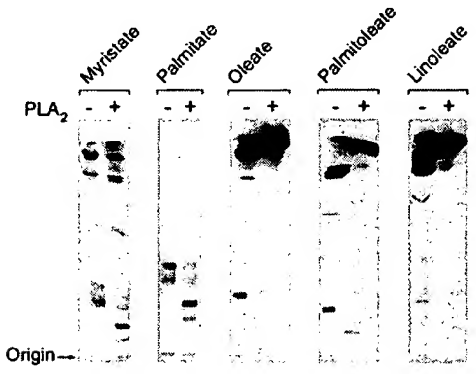

Figure 3. *P. falciparum* GPIs contain predominantly C18:1 fatty acid at *sn*-2. The parasites were metabolically labeled with various ^{3}H-fatty acids. Free GPIs were isolated, treated with bee venom phospholipase A_2 (PLA_2), and analyzed by HPTLC. −, untreated GPIs; +, phospholipase A_2–treated GPIs.

Treatment of [^{3}H]palmitoleic, [^{3}H]oleic, and [^{3}H]linoleic acid–labeled GPIs with phospholipase A_2 released >90% of the incorporated activity as free acid; the *lyso*-GPIs contained negligible radioactivity (Fig. 3). Treatment with HF showed that most of the incorporated radioactivity was on the diacylglycerol moiety, and the inositol carbohydrate moiety was not radioactive (not shown). These results agree with the presence of unsaturated fatty acyl residues at *sn*-2 as indicated by mass spectrometry of phospholipase A_2–treated GPIs. The incorporation of [^{3}H]palmitoleic acid to *sn*-2 is most likely after its conversion to cis-vaccenic acid; this agrees with the presence of cis-vaccenic acid at *sn*-2 of the GPIs (see above) and the ability of the parasite to synthesize this acid (42).

Based on the above data, the structure shown in Fig. 4 is proposed for *P. falciparum* GPIs.

TNF-α–inducing Activity of P. falciparum GPIs. Previous studies have shown that *P. falciparum* GPIs can induce the expression of proinflammatory cytokines in macrophages (27–29). To determine if highly purified GPIs can induce biologic activity, cultured murine macrophages were incubated with 0.03–0.5 μM purified GPIs, and the amount of TNF-α in the culture medium was measured. Consistent with a previous finding (26), the purified parasite GPIs induced TNF-α (30–450 pg/ml) in a dose-dependent manner (not shown).

Analysis for the Natural Anti-GPI Antibody in Humans. To examine whether people living in malaria endemic areas express naturally elicited anti-GPI antibodies, we analyzed sera from 300 adults in Western Kenya and 50 USA adults not exposed to malaria. In ELISAs performed with a coating of 2 ng/well of HPLC-purified, free GPIs, all Kenyan adult sera exhibited moderate to high levels of GPI-specific antibodies, predominantly IgGs, whereas all USA adults completely lacked anti-GPI antibodies (Fig. 5). The anti-GPI antibody activity of Kenyan sera was dose dependent and saturable (Fig. 5). There was no significant difference in levels of GPI-specific antibody reactivity between

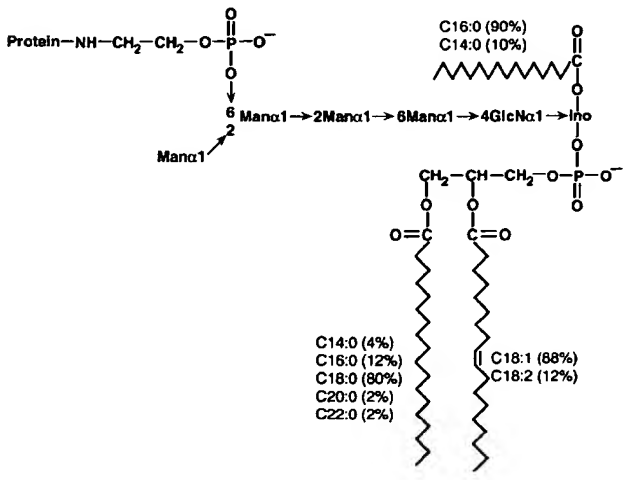

Figure 4. The proposed structures of *P. falciparum* GPIs. The fatty acids and their molar proportions are indicated.

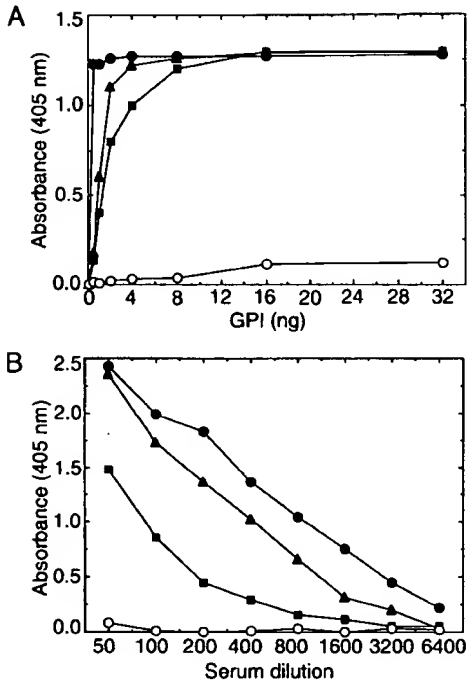

Figure 5. ELISA for naturally elicited anti-GPI antibodies in human sera. The HPLC-purified, free GPIs were coated onto 96-well microtiter plates either at the indicated amounts per well (A) or at 2 ng per well (B). The wells were blocked with TBS-casein, and then incubated with human sera, 1:100 or serially diluted with TBS-casein, 0.05% Tween 20. After washing the plates, the bound antibodies were detected with HRP-conjugated goat anti-human IgG (H and L chains) using ABTS substrate. ●, Kenyan adult sera 1; ▲, Kenyan adult sera 2; ■, Kenyan adult sera 3; O, USA adult control serum.

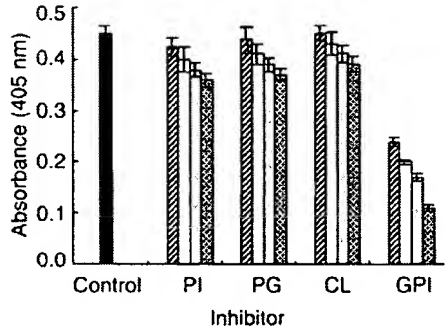

Figure 6. Inhibition of anti-GPI antibody binding to GPIs by phospholipids. The HPLC-purified, free GPIs were coated on to microtiter plates (2 ng), blocked with TBS-casein, and overlaid with representative Kenyan sera (1:200 diluted) incubated with the indicated phospholipids and purified GPIs. The bound antibodies were measured by HRP-conjugated goat anti-human IgG (H and L chains) using ABTS substrate. Shown are the data from a representative of 10 different sera analyzed. Black bar, without inhibitor (Control); hatched bars, 2.5 ng; white bars, 5 ng; gray bars, 10 ng; stippled bars, 20 ng.

HPLC-purified free and amino acid–linked GPIs, indicating that the immunoreactivity is not due to contamination by parasite proteins. More than 85% of Kenyan adult sera also contained lower but significant levels of GPI-specific IgM antibodies (Naik, R.S., unpublished results). The erythrocyte membrane debris, saponin lysate, and ghosts were nonreactive to the infected Kenyan sera (data not shown).

When ELISA was performed using several commercially available phospholipids, PIs from bovine liver and soybean that lack acyl substituent on inositol, PGs, and CL, Kenyan adult sera showed a low level of activity: ~5–15% of that observed with *P. falciparum* GPIs. At all coating concentrations tested (2–50 ng/well), these compounds showed similar low levels of antibody reactivity. Prior incubation of Kenyan sera with 2.5–20 ng/ml of PIs, PGs, or CL, and then ELISA using plates coated with HPLC-purified free GPIs showed in all cases ~5–15% lower antibody binding activity, irrespective of the concentrations of lipid used (Fig. 6). However, prior incubation with the parasite GPIs inhibited binding by up to 75% in a dose-dependent manner (Fig. 6). Together, these results suggest that Kenyan adult sera have low levels of reactivity to common acylated phosphoglycerols. This is not surprising because of the polyclonal nature of GPI-specific antibodies, which are expected to contain antibodies to the phosphoglycerol portion of GPIs that are common to this class of molecules.

TLC immunoblotting of the HPTLC-purified GPIs confirmed the specificity of the seroreactivity: adult Kenyan sera reacted with the GPIs, whereas control sera from USA adults were nonreactive (Fig. 7). Furthermore, the Kenyan adult sera were nonreactive to bovine liver and soybean PIs that lack an acyl substituent on inositol, PGs, and CL (Fig. 7, and data not shown); the apparent lack of reactivity to these lipids on TLC plates could be due to a low level of sensitivity. Furthermore, treatment of the GPIs with HNO_2 shifted the immunoreactivity to the position of the PI moiety, suggesting that this portion of the molecule is antigenic (Fig. 7).

The identity of the antigenic part of GPIs was further confirmed by inhibition of seroreactivity to intact GPIs using carbohydrate and lipid moieties of GPIs isolated after HNO_2 fragmentation. The carbohydrate moiety inhibited antibody binding by only ~5% at a 10-fold higher concentration compared with that of the coated, intact GPI. The lipid moiety, at the same coating concentration as that of intact GPIs, showed >70% antibody-binding activity, indicating that the PI moiety is the antigenic structure. These results suggest that acylated inositol is the major moiety that recognizes the naturally elicited anti-GPI IgGs.

Anti-GPI Antibody Response and Acquired Resistance to Malaria Pathogenesis. To determine whether the susceptibility of young children in malaria endemic areas is related to the absence of GPI-specific antibodies, sera taken every month after birth for 4 yr from a cohort of 48 children were analyzed (8). The results were compared with those of sera from 100 siblings (7–8 yr) and 50 nonpregnant mothers (20–25 yr) (Fig. 8 A). Malaria parasitemia and clinical parameters (hemoglobin and fever) were followed every 2 wk in this community-based cohort. Whereas a case-control study design compares extremes, this nonbiased design de-

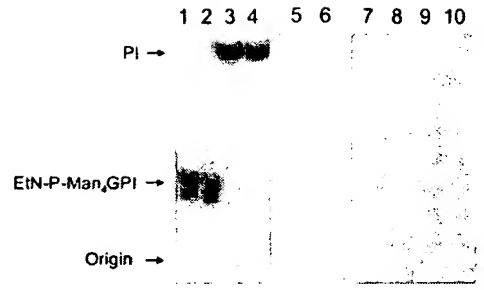

Figure 7. HPTLC immunochromatogram of *P. falciparum* GPIs. The HPTLC-purified, free GPIs (100 ng each) were chromatographed on HPTLC plates, blocked with 1% BSA, and incubated for 2 h in 1:100-diluted sera. The bound antibodies were detected with ^{125}I-labeled goat anti-human IgG (5 μCi/ml). Lanes 1 and 2, free and amino acid–linked GPIs, respectively, treated with Kenyan adult sera; lanes 3 and 4, HNO_2-released lipid from free and amino acid–linked GPIs, respectively, treated with Kenyan adult sera; lanes 5 and 6, free and amino acid–linked GPIs, respectively, treated with control USA adult sera; lanes 7 and 8, PIs from bovine liver and soybean, respectively; lane 9, PG; lane 10, CL, treated with Kenyan adult sera. Shown are representatives of 10 Kenyan and USA adult sera analyzed. Parasite lipids other than GPIs and their intermediates, extracted with chloroform/methanol (2:1, vol/vol), and glycolipids from control erythrocytes were completely nonreactive to Kenyan sera (not shown).

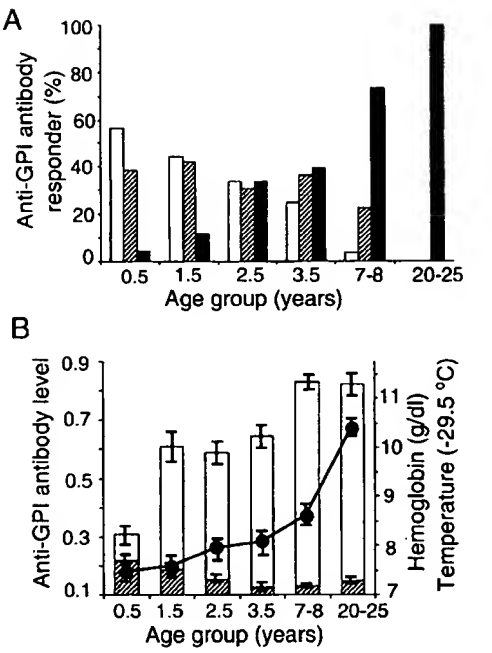

Figure 8. Age-dependent anti-GPI antibody response in people living in malaria endemic area. Sera from a cohort of children and adults were analyzed by ELISA using HPLC-purified, free GPIs (see Fig. 5). An antibody level (OD) greater than the mean of USA adult control sera plus 2 SD was considered positive. (A) Percentage of individuals in the following anti-GPI antibody responder categories: negative at both 1–3-mo-spaced time points sampled, negative (white bars); positive at one time point, intermittent (hatched bars); and positive at both time points, positive (black bars). $n \geq 40$ in each age group between ~0.5 and 3.5 yr, and $n = 100$ and 50 in the 7–8-yr and 20–25-yr age groups, respectively. A χ^2 test found that the antibody responder category was different among age groups ($P < 0.001$). (B) Mean hemoglobin (g/dl, white bars), temperature (−29.5°C, hatched bars), and anti-GPI antibody level [$\log_{10}$(OD+1)] (●) for the indicated age groups. Analysis of variance found that the anti-GPI antibody level, hemoglobin, and temperature were different among age groups ($P < 0.0001$).

termined if an association between anti-GPI antibodies and malaria pathogenesis was detectable at a population level. We carefully controlled for *P. falciparum* transmission, antimalaria drug treatment, and age. By sampling the same child over 4 yr, we found that, within an individual, anti-GPI antibody responses correlate with protection against malaria-attributable hemoglobin loss and febrile illness (see below).

We performed ELISA on sera from each child on all monthly samples and for siblings and adults at two time points spaced 1–3 mo apart to determine the anti-GPI antibody level and the persistence of the antibody response. For comparison and correlation analysis, results of four time points (0.5, 1.5, 2.5, and 3.5 yr old), based on the sampling scheme employed for the 7–8-yr-olds and the mothers, were used for children (Fig. 8 A). The data show that ~50% of the children under 2 yr lacked anti-GPI antibodies (negative responders, defined in the legend to Fig. 8), ~40% had short-lived antibody responses (lasted in the circulation for only 1–3 mo, intermittent responders), and only ~10% had persistent (long-lived) antibody responses (positive responders) (Fig. 8 A). In contrast, ~75% of the 7–8-yr-old children and all adults exhibited a persistent anti-GPI antibody response. The level of anti-GPI IgG increased with increasing age from infancy to adulthood, whereas mean hemoglobin density increased and fever (temperature) decreased with age up to 7–8 yr of life (Fig. 8 B). Thus, the presence of long-lived, high levels of anti-GPI antibodies in sera parallels the naturally acquired resistance against malaria pathogenesis.

We found that anti-GPI antibody response, age, and parasite density were independently associated with hemoglobin ($P < 0.0147$). Age influenced the anti-GPI antibody responder category; however, it was not significant ($P < 0.0581$), suggesting that the association between the antibody responder category (as defined in the legend to Fig. 8) and hemoglobin was not just a reflection of age. For example, using a general linear model, it was estimated that 6-mo-old children in the positive antibody responder category had 2.37 g/dl of hemoglobin more than 6-mo-olds in the intermittent responder category. Antibody responder category and parasite density were independently associated with temperature ($P < 0.0012$). 6-mo-olds in the negative antibody responder category had an estimated temperature 0.71°C higher than the individuals in the intermittent antibody responder category. Fig. 9 shows the associations between anti-GPI antibody responder category and malaria-attributable pathology in children 0.5–3.5 yr old. In each parasitemia category, febrile illness increased and hemoglobin level decreased in children without antibodies or with only short-lived antibodies; however, in children with persistent antibodies, febrile illness was lower and hemoglobin level was higher. These results strongly support the hypothesis that circulating anti-GPI antibodies neutralize the toxic effects of parasite GPIs.

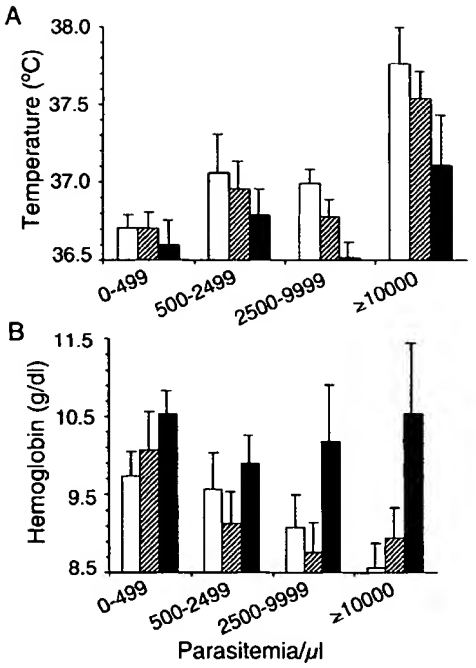

Figure 9. Serum anti-GPI antibody response and resistance to malaria in children 0.5–3.5 yr of age. A general linear model (GLM) was used to investigate the correlation between anti-GPI antibody responder category and temperature or hemoglobin level, while controlling for age and parasite density. (A) Mean temperature (°C) for 0.5–3.5-yr-old negative (white bars), intermittent (hatched bars), and positive (black bars) anti-GPI antibody responder categories. (B) Mean hemoglobin (g/dl) for 0.5–3.5-yr-old negative (white bars), intermittent (hatched bars), and positive (black bars) anti-GPI antibody responder categories (see legend to Fig. 8). The anti-GPI antibody responder category and parasite density were independently associated with hemoglobin ($P < 0.0147$ and $P = 0.0000$, respectively). The associations were independent of the nonsignificant association between age and hemoglobin ($P = 0.0581$). Antibody responder category and parasite density were independently associated with temperature ($P = 0.0012$ and 0.0001, respectively). Age was not found significantly correlated with temperature ($P > 0.0581$).

Discussion

This paper reports two major findings. (a) The results presented here establish for the first time that individuals residing in malaria-endemic areas develop a *P. falciparum*–specific anti-GPI antibody response, whereas people not exposed to the malaria parasite do not have these antibodies; in addition, anti-GPI antibody responses are correlated with protection against malaria-related febrile illness and hemoglobin loss. This agrees with the previous reports describing *P. falciparum* GPIs as pathogenicity factors based on their ability to induce a spectrum of proinflammatory cytokine responses and cause malaria symptoms. (b) Another important finding is elucidation of complete structures of the parasite GPIs by direct biochemical analysis and mass spectrometry. Thus, our results identified two novel structural features of *P. falciparum* GPIs: the presence of palmitate and myristate on C-2 of inositol, and the presence of predominantly oleic and minor amounts of cis-vaccenic acid and C18:2 at *sn*-2.

The purification of *P. falciparum* GPIs to homogeneity was crucial for structural determination as well as to obtain unambiguous evidence for biologic activity and establish the presence and specificity of naturally elicited antibodies in the sera of people living in malaria endemic areas. The key steps employed for isolation and purification of GPIs were: (a) growing of mycoplasma-free cultures to high levels of parasitemia and enrichment of infected erythrocytes; (b) metabolic labeling of GPIs and the use of human sera containing anti-GPI antibodies to follow purification steps; (c) use of sterile water and buffers and high quality organic solvents to exclude external contamination; and (d) siliconizing glassware to avoid loss due to surface adsorption. The mass spectrometry results presented here show that the purified GPIs are homogeneous.

We determined the structures of *P. falciparum* GPIs by subjecting the [^{3}H]GlcN- and ^{3}H–fatty acid-labeled GPIs to various standard degradative procedures, including their susceptibility to nitrous acid, HF, alkali, jack bean α-mannosidase, and GPI-specific phospholipase D, by determining fatty acid composition and finally by direct mass spectrometry. The results enabled us to propose the structures shown in Fig. 4 for the parasite GPIs. The structure of the core glycan is the same as determined previously (19, 20). However, the acyl substituents are different from those reported previously, based on radiolabeling, with palmitate at both *sn*-1 and *sn*-2 and predominantly myristate on inositol (21). As shown in Fig. 4, palmitate is the major acyl substituent with minor proportions of myristate on inositol of the parasite GPIs; GPIs with acylated inositol residues from other sources reported to date contain only palmitate on the inositol residue (12). The parasite GPIs contain unsaturated acyl substituents at *sn*-2 (major C18:1 and minor C18:2) and predominantly C18:0 and a range of variable size saturated acyl residues at *sn*-1. With respect to the nature of the acyl residue at *sn*-2, the parasite GPIs resemble the GPI of *T. cruzi* trypomastigote mucin that has a potent cytokine-inducing property (30). Another unusual feature of the parasite GPIs is the presence of cis-vaccenic acid at *sn*-2.

The parasite GPIs differ significantly from those of humans with respect to both the acyl substituents and the carbohydrate moiety (12). The GPI moieties of human erythrocyte proteins (the erythrocytes do not contain detectable levels of free GPIs), CD59, and acetylcholine esterase contain exclusively a C18 alkyl substituent at *sn*-1, C22:4 at *sn*-2, and palmitate on inositol; the carbohydrate moieties contain one or two extra phosphoethanolamine as well as βGalNAc residues (12). The GPI of human spleen CD52 contains a diacylglycerol moiety and lacks GalNAc; however, this differs from the parasite GPIs with respect to the type of fatty acid at *sn*-2 (C22:4, C22:5, and C22:6) and contains phosphoethanolamine on the first mannose residue (12). These structural differences may contribute to the observed naturally elicited immunologic responses against the parasite GPIs in humans.

Although Schofield et al. have shown that *P. falciparum* GPIs can transduce signals to elicit inflammatory cytokine

responses (27–29), there have been concerns as to whether the observed activity was due to contamination (parasite, erythrocytes, and/or mycoplasma origin [43]). These concerns were based on the observations by some investigators that aqueous buffer extracts of parasite cultures, presumed to have extracted the parasite GPIs upon boiling, could not elicit TNF-α. Thus, they have argued that the cytokine-inducing property of *P. falciparum* was due to unknown components. However, it should be noted that GPIs can only be extracted with organic solvents. Because of this existing controversy, we tested TNF-α induction by the highly purified parasite GPIs. The results presented in this paper clearly show that the purified *P. falciparum* GPIs can induce TNF-α in macrophages. This activity is consistent with the recent finding by Almeida et al. that a highly purified GPI moiety of *T. cruzi* trypomastigote mucin induces TNF-α (30) and confirms the previous finding by Schofield et al. (26). Furthermore, studies by Almeida et al. also show that C18:1 and/or C18:2 acyl substituent at *sn*-2 in the *T. cruzi* GPIs is critical for TNF-α–inducing activity (30). As the *P. falciparum* GPI also contains C18:1 (major) and C18:2 (minor) at *sn*-2, it is possible that these acyl substituents contribute to the toxic property of the parasite GPIs.

In malaria endemic areas, younger children have the highest risk of developing severe malaria, whereas older children and adults rarely develop severe disease despite repeated exposure and significant parasitemia (23). People in nonmalarious regions completely lack this resistance, suggesting that the protection is due to a parasite-specific response acquired through repeated infections. In the Western Kenyan population studied here, we found that adults have *P. falciparum* parasitemia >14% of the time, whereas children <4 yr old have parasitemia >60% of the time. The density of parasitemia is lower in adults and, importantly, the level of parasitemia that can be tolerated without causing febrile illness or anemia is higher in adults (Branch, O.H., unpublished). As *P. falciparum* GPIs are pathogenicity factors (26–29), the resistance of adults to malaria illness may be related to a GPI-specific protective immunity.

All the Kenyan adult sera analyzed contained high levels of GPI-specific IgGs, whereas all 50 USA adult sera completely lacked such antibodies. The antibody response was highly specific to GPIs and their intermediates; other phospholipids of the parasite showed only low levels of immunoreactivity. Several phospholipids, including PIs, PGs, and CL, showed only 5–15% of the immunoreactivity exhibited by the parasite GPIs, which appears to be due to the polyclonal nature of the antibodies reacting with the common epitopes. Previously, several studies have reported the presence of significant levels of antibodies that bind phospholipids either directly or via binding of serum β2-glycoprotein I (44). In those studies, lipids were coated with several micrograms per well for ELISA. It is known that proteins and antibodies can nonspecifically bind to lipids when coated at high density. In contrast, our study used 0.5–2 ng/well of GPIs for ELISA and 100 ng for TLC immunoblots. Moreover, in previous studies, plates coated with lipid antigens were incubated with sera diluted with buffers without detergent. Under such conditions, we found high levels of nonspecific activity. Thus, our study clearly demonstrates that the identified IgGs are specific to GPIs.

Our results also establish that the PI portion of the GPIs contributes significantly to immunogenicity. The removal of the *sn*-2 fatty acid from the GPIs did not affect antibody reactivity (Vijaykumar, M., unpublished results). As treatment with HF abolished immunoreactivity, it appears that the acylated inositol phosphate is the immunogenic portion of the molecule. This agrees with the previous finding that antibodies raised against PIs can inhibit the induction of TNF-α by *P. falciparum* extracts (45). These results are important in that if an *sn*-2 acyl substituent is indeed required for cytokine-inducing activity, then it may be possible to synthesize nontoxic molecules for therapeutic purposes.

The TNF-α–inducing activity of GPIs and the correlation between the general resistance of adults in endemic areas to malaria pathogenesis and the presence of serum anti-GPI antibody response suggest that the acquired immunity is related significantly to the anti-GPI antibodies. This prediction agrees with the lack of such an antibody response in the majority of children <4 yr old, the risk of children developing severe malaria, and the correlation between the gradual acquisition of the antibody response in an age-dependent manner (>80% of the 7–8-yr-olds having high levels of serum antibody) and protection against malaria with age. Thus, a direct correlation between anti-GPI antibodies and malaria-related pathology could be observed in young children.

High levels of antibodies against several other antigens such as MSP-1, EBA-175, and circumsporozoite protein are also present in adults, and therefore it may be argued that the anti-GPI antibody response is not independent from those antibodies. However, it is important to consider that the anti-GPI antibody response is related to antidisease immunity, whereas antibodies against parasite proteins studied to date are involved in antiparasite immunity (controlling parasite burden). Furthermore, whereas ~80% of children <2 yr of age either lack or contain very low levels of short-lived anti-GPI antibodies, these children can have high levels of antibodies against MSP-1 and other proteins (8, 9; and Branch, O.H., unpublished results). Although there was a correlation between the levels of MSP-1 and EBA-175 antibodies and protection against parasite density, these antibody responses were not related to anti-GPI antibody response, protection against febrile illness, and hemoglobin loss at any given parasite density.

Almost all malaria vaccine development efforts currently being pursued use parasite proteins in a multicomponent formulation, aimed at providing immunity against infection (antiparasitic [5]). On the other hand, by blocking the toxic effects of the parasite GPIs, a GPI-based vaccine might significantly reduce malaria pathogenesis. The identification of the PI moiety as the functional part of the molecules should significantly simplify any approach towards the development of antidisease measures.

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References

1. Oaks, S.C., Jr., V.S. Mitchell, G.W. Pearson, and C.C.J. Carpenter, editors. 1991. Malaria: Obstacles and Opportunities. National Academy Press, Washington, DC. 309 pp.
2. Holder, A.A. 1999. Malaria vaccines. *Proc. Natl. Acad. Sci. USA.* 96:1167–1169.
3. Doolan, D.L., and S.L. Hoffman. 1997. Multi-gene vaccination against malaria: a multistage, multi-immune response approach. *Parasitol. Today.* 13:171–178.
4. Ockenhouse, C.F., P.F. Sun, D.E. Lanar, B.T. Wellde, B.T. Hall, K. Kester, J.A. Stoute, A. Magill, U. Krzych, L. Farley, et al. 1998. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J. Infect. Dis.* 177:1664–1673.
5. Shi, Y.P., S.E. Hasnain, J.B. Sacci, B.P. Holloway, H. Fujioka, N. Kumar, R. Wohlhueter, S.L. Hoffman, W.E. Collins, and A.A. Lal. 1999. Immunogenicity and *in vitro* protective efficacy of a recombinant multistage *Plasmodium falciparum* candidate vaccine. *Proc. Natl. Acad. Sci. USA.* 96: 1615–1620.
6. Baird, J.K. 1995. Host age as a determinant of naturally acquired immunity to *Plasmodium falciparum* malaria. *Parasitol. Today.* 11:105–111.
7. McGuinness, D., K. Koram, S. Bennett, G. Wagner, F. Nkrumah, and E. Riley. 1998. Clinical case definitions for malaria: clinical malaria associated with very low parasite densities in African infants. *Trans. R. Soc. Trop. Med. Hyg.* 92: 527–531.
8. Branch, O.H., A.J. Oloo, B.L. Nahlen, D.C. Kaslow, and A.A. Lal. 2000. Anti-merozoite surface protein-1 19 kDa IgG in mother-infant pairs: subclass analysis with age, exposure, and protection against *Plasmodium falciparum* malaria. *J. Infect. Dis.* 181:1746–1752.
9. Branch, O.H., V. Udhayakumar, A.W. Hightower, A.J. Oloo, W.A. Hawley, B.L. Nahlen, P.B. Bloland, D.C. Kaslow, and A.A. Lal. 1998. A longitudinal investigation of IgG and IgM antibody response to the merozoite surface protein-1 19 kilodalton domain of *Plasmodium falciparum* in pregnant women and infants: association with febrile illness, parasitemia, and anemia. *Am. J. Trop. Med. Hyg.* 58:211–219.
10. McConville, M.J., and M.A.J. Ferguson. 1993. The structure, biosynthesis, and function of glycosylated phosphatidylinositols in the parasite protozoa and higher eukaryotes. *Biochem. J.* 294:305–324.
11. Englund, P.T. 1993. The structure and biosynthesis of glycosylphosphatidylinositol protein anchors. *Annu. Rev. Biochem.* 62:121–138.
12. Ferguson, M.A.J., J.S. Brimacombe, J.R. Brown, A. Crossman, A. Dix, R.A. Field, M.L. Guther, K.G. Milne, D.K. Sharma, and T.K. Smith. 1999. The GPI biosynthetic pathway as a therapeutic target for African sleeping sickness. *Biochim. Biophys. Acta.* 1455:327–340.
13. Miller, L.H., T. Roberts, M. Shahabuddin, and T.F. McCutchan. 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Mol. Biochem. Parasitol.* 59:1–14.
14. Smythe, J.A., R.L. Coppel, G.V. Brown, R. Ramasamy, D.J. Kemp, and R.F. Anders. 1988. Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA.* 85:5195–5199.
15. Marshall, V.M., A. Silva, M. Foley, S. Cranmer, L. Wang, D.J. McColl, D.J. Kemp, and R.L. Coppel. 1997. A second merozoite surface protein (MSP-4) of *Plasmodium falciparum* that contains an epidermal growth factor-like domain. *Infect. Immun.* 65:4460–4467.
16. Braun-Breton, C., T.L. Rosenberry, and L.H. Pereria da Silva. 1990. Glycolipid anchorage of *Plasmodium falciparum* surface antigens. *Res. Immunol.* 141:743–755.
17. Haldar, K., C.L. Henderson, and G.A.M. Cross. 1986. Glycolipid anchorage of *Plasmodium falciparum* surface antigens. *Proc. Natl. Acad. Sci. USA.* 83:8565–8569.
18. Naik, R.S., E.A. Davidson, and D.C. Gowda. 2000. Developmental stage-specific biosynthesis of glycosylphosphatidylinositol anchors in intraerythrocytic *Plasmodium falciparum* and its inhibition in a novel manner by mannosamine. *J. Biol. Chem.* 275:24506–24511.
19. Gowda, D.C., P. Gupta, and E.A. Davidson. 1997. Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage *Plasmodium falciparum*. *J. Biol. Chem.* 272:6428–6439.
20. Gerold, P., A. Dieckmann-Schuppert, and R.T. Schwarz. 1994. Glycosylphosphatidyl-inositols synthesized by asexual erythrocytic stages of the malarial parasite, *Plasmodium falciparum*. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. *J. Biol. Chem.* 269:2597–2606.
21. Gerold, P., L. Schofield, M.J. Blackman, A.A. Holder, and R.T. Schwarz. 1996. Structural analysis of the glycosylphosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 75:131–143.
22. Playfair, J.H.L, J. Taverne, C.A. Bate, and J.B. de Souza. 1990. The malaria vaccine: anti-parasite or anti-disease? *Immunol. Today.* 11:25–27.
23. Hommel, M. 1993. Amplification of cytoadherence in cerebral malaria: towards a more rational explanation of disease. *Ann. Trop. Med. Parasitol.* 87:627–635.
24. Kwiatkowski, D., A.V. Hill, I. Sambou, P. Twumasi, J. Castracane, K.R. Manogue, A. Cerami, D.R. Brewster, and B.M. Greenwood. 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet.* 336:1201–1204.
25. Grau, G.E., L.F. Fajardo, P.F. Piguet, B. Allet, P.H. Lambert, and P. Vassalli. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science.* 237: 1210–1212.

26. Schofield, L., and F. Hackett. 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. *J. Exp. Med.* 177:145–153.
27. Schofield, L., L. Vivas, F. Hackett, P. Gerold, R.T. Schwarz, and S. Tachado. 1993. Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-α-inducing toxin of *Plasmodium falciparum*: prospects for the immunotherapy of severe malaria. *Ann. Trop. Med. Parasitol.* 87:617–626.
28. Schofield, L., S. Novakovic, P. Gerold, R.T. Schwarz, M.J. McConville, and S.D. Tachado. 1996. Glycosylphosphatidylinositol toxin of *Plasmodium* up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J. Immunol.* 156:1886–1896.
29. Tachado, S.D., P. Gerold, R. Schwarz, S. Novakovic, M. McConville, and L. Schofield. 1997. Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania:* activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. *Proc. Natl. Acad. Sci. USA.* 94:4022–4027.
30. Almeida, I.C., M.M. Camargo, D.O. Procopio, L.S. Silva, A. Mehlert, L.R. Travassos, R.T. Gazzinelli, and M.A.J. Ferguson. 2000. Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:1476–1485.
31. Lambros, C., and J.P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65:418–420.
32. Rowe, J.A., I.G. Scragg, D. Kwiatkowski, D.J. Ferguson, D.J. Carucci, and C.I. Newbold. 1998. Implications of mycoplasma contamination in *Plasmodium falciparum* cultures and methods for its detection and eradication. *Mol. Biochem. Parasitol.* 92:177–180.
33. Jensen, J.B. 1978. Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* 27:1274–1276.
34. Trager, W. 1959. The enhanced folic and folinic acid contents of erythrocytes infected with malaria parasites. *Exp. Parasitol.* 8:265–273.
35. Udeinya, I.J., and K. Van Dyke. 1980. Labeling of membrane glycoproteins of cultivated *Plasmodium falciparum*. *Bull. World Health Organ.* 58:445–448.
36. Roberts, W.L., S. Santikarn, V.N. Reinhold, and T.L. Rosenberry. 1988. Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase by fast atom bombardment mass spectrometry. *J. Biol. Chem.* 263:18776–18784.
37. Hardy, M.R., and R.R. Townsend. 1994. High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates. *Methods Enzymol.* 230:208–225.
38. Woods, A.S., A.Y. Huang, R.J. Cotter, G.R. Pasternack, D.M. Pardoll, and E.M. Jaffee. 1995. Simplified high-sensitivity sequencing of a major histocompatibility complex class I-associated immunoreactive peptide using matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* 226:15–25.
39. Magnani, J.L., S.L. Spitalnik, and V. Ginsburg. 1987. Antibodies against cell surface carbohydrates: determination of antigen structure. *Methods Enzymol.* 138:195–207.
40. Ferguson, M.A.J. 1992. Chemical and enzyme analysis of glycosylphosphatidylinositol anchors. *In* Lipid Modification of Proteins: A Practical Approach. N.M. Hooper and A.J. Turner, editors. IRL Press, New York. 191–231.
41. Zeger, S.L., and K.Y. Liang. 1986. Longitudinal data analysis for discrete and continuous outcomes. *Biometrics.* 42:121–130.
42. Holz, G.G., Jr. 1977. Lipids and the malarial parasite. *Bull. World Health Organ.* 55:237–248.
43. Taverne, J., N. Sheikh, K. Elased, J. Playfair, M. Scott, and D. Saggerson. 1997. Malaria toxins revisited—reply. *Parasitol. Today.* 13:276–277.
44. Jakobsen, P.H., V. McKay, R. N'Jie, B.O. Olaleye, U. D'Alessandro, K. Bendtzen, I. Schousboe, and B.M. Greenwood. 1996. Soluble products of inflammatory reactions are not induced in children with asymptomatic *Plasmodium falciparum* infections. *Clin. Exp. Immunol.* 105:69–73.
45. Bate, C.A.W., and D. Kwiatkowski. 1994. A monoclonal antibody that recognizes phosphatidylinositol inhibits induction of tumor necrosis factor alpha by different strains of *Plasmodium falciparum*. *Infect. Immun.* 62:5261–5266.

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Original article

Differential antibody responses to *Plasmodium falciparum* glycosylphosphatidylinositol anchors in patients with cerebral and mild malaria

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Abstract

Glycosylphosphatidylinositol (GPI) membrane anchors of *Plasmodium falciparum* surface proteins are thought to be important factors contributing to malaria pathogenesis, and anti-GPI antibodies have been suggested to provide protection by neutralizing the toxic activity of GPIs. In this study, IgG responses against *P. falciparum* GPIs and a baculovirus recombinant MSP1p19 antigen were evaluated in two distinct groups of 70 patients each, who were hospitalized with malaria. Anti-GPI IgGs were significantly lower in patients hospitalized with confirmed cerebral malaria compared to those with mild malaria ($P < 0.01$) but did not discriminate for fatal outcome. In contrast, a specific marker of the anti-parasite immunity, as monitored by the anti-MSP1p19 IgG response, was similar in both cerebral and mild malaria individuals, although it was significantly lower in a subgroup with fatal outcomes. These results are consistent with a potential anti-toxin role for anti-GPI antibodies associated with protection against cerebral malaria.

Keywords: *Plasmodium falciparum*; Glycosylphosphatidylinositol anchors; MSP1p19; Antibody response; Cerebral malaria; Protection

1. Introduction

Many clinical manifestations of *Plasmodium falciparum* infection are caused by a complex cascade of events triggered during schizont rupture. Glycosylphosphatidylinositol (GPI) anchor structures are considered to be important parasite toxin candidates that could contribute to immunopathological events, leading to the development of severe malaria. The patho-physiological effects of parasite GPIs have been attributed to their ability to induce the production of proinflammatory cytokines in the host, including tumor necrosis factor (TNF-α), interleukin-1 (IL1), nitric oxide (NO), and interferon (IFN-γ) [1–4]. Additionally, *Plasmodium* GPIs have been proposed to mediate hypoglycemia by mimicking the activity of insulin [5].

GPIs are ubiquitous in eukaryotes, and are primarily involved in anchoring certain cell surface proteins to plasma membranes. Compared to animal cells, GPIs are abundantly expressed in various parasite species including *Plasmodium*, *Trypanosoma* and *Leishmania*, and these organisms contain large pools of free GPIs that are not attached to proteins [6]. Unlike protein anchored forms, free GPIs are not masked on cell surfaces, and therefore are more accessible for triggering innate immune responses [7], including pro-inflammatory cytokine secretion [8]. Although the physiological functions of the GPIs remain poorly understood, it appears likely that the parasites use GPI bioactivity to stimulate host immune responses for their own benefit. However, uncontrolled stimulation of the innate immune system is deleterious to host, and that can lead to severe clinical symptoms [7].

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Since individuals living in areas of high malaria transmission have acquired immunity to malaria pathogenesis, anti-GPI antibodies have been proposed as mediators of malaria "anti-disease" immunity. Several studies have addressed the question of the protective role of anti-GPI antibodies [8–11]. In malaria endemic areas, anti-GPI IgG are produced in an age-dependent manner, correlating with the cumulative age-related acquisition of protective immunity to malaria. It has also been observed that antibodies to GPIs are predominantly of the IgG3 subclass and are rapidly boosted in response to infection, but they are short-lived [9–12]. While a recent study has shown that anti-GPI antibodies are higher in children with asymptomatic infections compared to those exhibiting clinical symptoms [9,10,13], the observed differences were not statistically significant and there is clearly a need for more controlled studies.

On the other hand, acquired immunity relates to IgG responses against protection-associated target Ags with effective anti-parasite activity such as the conserved 19 kDa C-terminal fragment of the 200-kDa major merozoite surface protein 1. During merozoite invasion of erythrocytes, a major portion of N-terminal MSP1 is proteolytically cleaved, leaving PfMSP1p19 anchored to the parasite surface by a GPI moiety that is carried into newly invaded erythrocytes [14]. Antibody responses to PfMSP1p19 have been extensively studied and shown to be associated with clinical immunity in children and adults [15,16]. PfMSP1p19 is known to induce an effective *P. falciparum* anti-parasite immune response by inducing antibodies that interfere with the merozoite invasion process [17].

We investigated, in a case–control study consisting of individuals from an urban area with symptoms of cerebral malaria at hospital in Dakar, Senegal, the potential protection-associated role of *P. falciparum* anti-GPI IgG responses in malaria pathogenesis. Two sets of distinct, well-defined groups of patients with confirmed cerebral malaria were recruited in two consecutive timeframes. In parallel, as a marker of IgG response to infection, we measured the levels of anti-MSP1 IgGs using a recombinant PfMSP1p19 expressed in baculovirus [18]. The recombinant PfMSP1p19 antigen is strongly recognized by the sera of infected individuals, and IgG responses were shown to be significantly associated with delayed infection following drug cure [19], and with clinical protection [20]. Our results show that, patients with cerebral malaria had significantly lower levels of anti-GPI antibodies compared to control group of individuals hospitalized with mild malaria and that anti-GPI antibody response do not relate with fatal outcome.

2. Materials and methods

2.1. Sample collection and study population

Subjects were patients, treated at Hopital Principal, living in the hypoendemic urban area of Dakar (Senegal). In Dakar, over 2 million people seasonally receive an average of 0.5 infective bite per individual per year, with highly variable densities of vector anopheline mosquitoes [21]. A mean incidence of 2.4% of clinical accesses (26 cases out of 1067) were observed [13]. Blood samples collected for biological investigations from patients hospitalized for acute symptoms of malaria at different periods of time, recruited yearly from September to December, were used.

The study group 1 consisted of 70 hospitalized "adults" (≥13 years old, and not treated in the pediatric intensive care facilities). In this group, 35 patients had confirmed cerebral malaria (sCM) and recovered with no sequelae (mean age 28 years, sampled in 1998–1999), and 35 adults hospitalized for "mild" malaria (mean age 31.7 years, sampled in November–December 1999) (hMM). In these two groups, there was no significant difference in the age distribution (Table 1).

The study group 2 consisted of 70 patients hospitalized with cerebral malaria, sampled in 2000 and 2001. Of these, 24 were aged 13–63 years (mean age 31 years), 28 were 2–12 years old (mean age 6.8 years) with confirmed cerebral malaria but recovered (sCM), and 18 adults and children 2.6–63 years old had a fatal outcome to cerebral malaria (fCM, see Table 1). In this study, we included two control groups: (i) 30 individuals treated for "mild" malaria (MM) at the health center of Dakar (6–46 years, mean 17.3 years); (ii) 47 uninfected individuals (2–62 years, mean 20.6 years) living in Dakar (NI), sampled in the context of routine biological analyses carried out at the hospital during the transmission period. The NI group was included to check for negativity of anti-GPI Ab in individuals without recent history of infection

Table 1
Characteristics, levels and prevalence of IgG responses against MSP1p19 and GPI in the different study groups

Study	Period	Patient class	N_i	age groups <13	age groups ≥13	age mean±SD	Ab to GPI[4] %	Ab to GPI[4] med. [Range]	Ab to GPI[4] mean	Ab to MSP1[4] %	Ab to MSP1[4] med. [Range]	Ab to MSP1[4] mean
1	1998-99	sCM[1]	35	-	35	27.0 [14.6]	17	1.1 [1-9.7]	1.7	94	8.0 [1.8-10.4]	7.2
1	1999	hMM[2]	35	-	35	31.7 [15.5]	31	1.5 [1-17.1]	2.6	100	8.4 [2.6-9.9]	7.7
2	2000-01	sCM[1]	52	28	24	18.0 [16.4]	15	1.1 [1-6.1]	1.6	79	8.0 [1-13.1]	7.4
2	2000-01	fCM[1]	18	7	11	20.6 [13.9]	11	1.2 [1-2.9]	1.4	50	2.4 [1-12.5]	4.7
2	2000	MM[3]	30	12	18	16.4 [9.6]	33	1.2 [1-13.5]	2.8	93	7.5 [1.1-18.9]	7.5
2	2001	NI[1]	47	20	27	20.5 [16.0]	0	1 [1-1.9]	1.1	28	1 [1-8.7]	1.7

[1]sCM=surviving cerebral malaria; fCM = fatal cerebral malaria; NI = non infected individuals
[2]hMM=hospitalized mild malaria controls in study 1
[3]MM=mild malaria controls from health center in study 2
[4]Ab responses are expressed as %=incidence of responses; med.=median value; range and mean values of OD ratio

(Table 1). There was no significant difference in the age distribution among the three age-groups.

After collection of blood samples, red blood cells were separated by centrifugation, and plasma stored at –20 °C until used. Each set of samples has been grouped and analyzed separately.

2.2. Antigens and ELISA procedure

GPIs were isolated and purified by HPLC as described [9]. Recombinant PfMSP1p19 (Palo Alto allele), was produced in *Trichoplusia ni* insect cells (High Five, Invitrogen) infected with recombinant baculovirus and purified by metalloaffinity chromatography [22]. GPIs were dissolved in methanol and 50 µl per well (2 ng GPI) was transferred to flat-bottomed Immulon-4 96-well microtiter plates (Dynatech, Springfield, VA). Plates were dried at 37 °C and blocked with Phosphate-buffered saline containing 5% BSA (PBS-BSA). Microtiter plates were coated with 100 µl per well of PfMSP1p19 at a concentration of 0.5 µg/ml. Plasma samples were diluted 1:100 in PBS with 1% BSA /0.05% Tween 20, and ELISAs were performed as described previously [9,19,23].

The results are expressed as OD ratios, i.e. $OD_{sample}/OD_{negative\ control}$. The negative control was pooled plasma from Europeans not exposed to malaria. Positive responders were individuals with OD ratios > 2 (corresponding to mean OD + 2 S.D. of naive controls) [19,23]. For positive controls, ODs were ~0.45 (OD ratio of ~6) and ~1.6 (OD ratio of ~11) for GPI and PfMSP1p19, respectively.

2.3. Statistical analysis

Comparisons of antibody levels between different groups were done by the Mann–Whitney rank test and the Spearman rank correlation test for non-normally distributed data. The Fisher's exact test and the chi-square were used to compare prevalence of responses between groups. P values < 0.05 were considered significant after the Bonferonni correction for multiple comparison. Statistical analyses were performed using Stata 7.0 and Statview 5.0® software (SAS Institute, Cary, NJ).

3. Results

3.1. IgG responses to PfMSP1p19 and GPI in study group 1

As summarized in Table 1, in this study group, MSP1p19 was strongly recognized by all patients (94–100% responders with an OD ratio > 2). There were similar levels and distribution of PfMSP1p19 specific IgG in groups with mild (hMM, median = 8.4), or cerebral malaria (sCM, median = 8.0) (Fig. 1a). In contrast, the anti-GPI IgG levels were significantly lower ($P < 0.01$) in individuals with sCM (median = 1.1) compared to hMM (median = 1.5) (Fig. 1b). The distribution of anti-GPI antibody responses was different between sCM and hMM cases; fewer individuals showing elevated values in sCM. Further, the prevalence of responders was significantly lower in sCM compared with hMM (chi square = 5.51; $P = 0.02$).

There was no significant correlation between IgG responses against MSP1p19 and GPI (Fig. 2a). Only a few individuals with cerebral malaria had high responses to both antigens. Importantly, there was no correlation between age of individuals and IgG responses against PfMSP1p19 or GPI in sCM and hMM groups. Additional control using samples from individuals living in an holoendemic setting (data not shown), exhibited age-associated IgG responses, in agreement with previous studies, showing that the anti-GPI IgG responses increased with age, along with cumulative immune responses in individuals continuously exposed to *P. falciparum* [11].

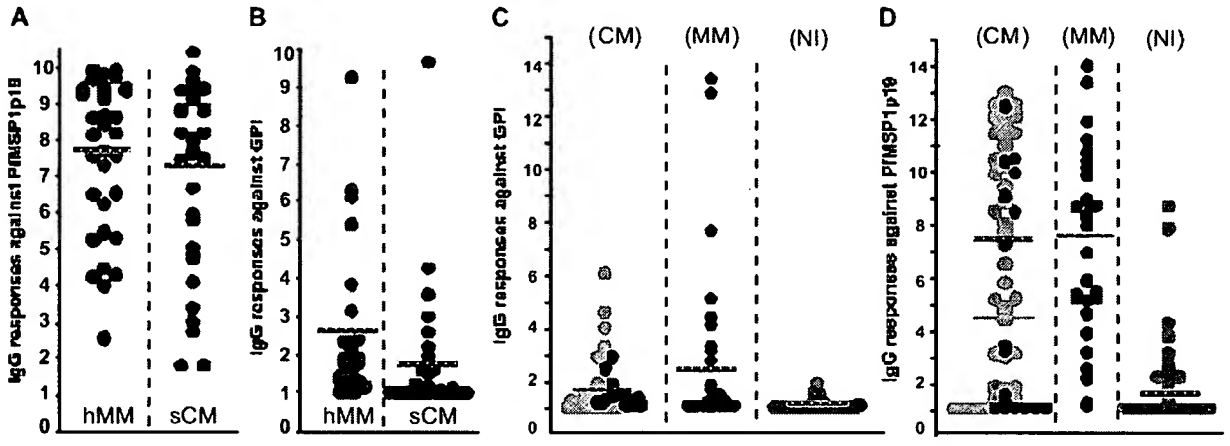

Fig. 1. Scatter plot of the distribution of antibodies against GPIs and MSP1p19 in the different study groups. Shown are IgG responses against PfMSP1p19 (**a, d**) and GPI (**b, c**) for individuals in study group 1 (**a, b**) and study group 2 (**c, d**). hMM and hCM, respectively, represent 35 mild malaria and 35 cerebral malaria cases in the study group 1. MM, CM and NI, respectively, represent mild malaria cases from the Health Center, cerebral malaria cases, and non-immune controls in the study group 2. Fatal cases among CM individuals of study group 2 (**c, d**) are plotted in black. Mean levels of IgG response in **a–d** are indicated by black horizontal lines and mean anti-pfMSP1p19 level of fatal cases (**d**) is indicated by gray line (**c**).

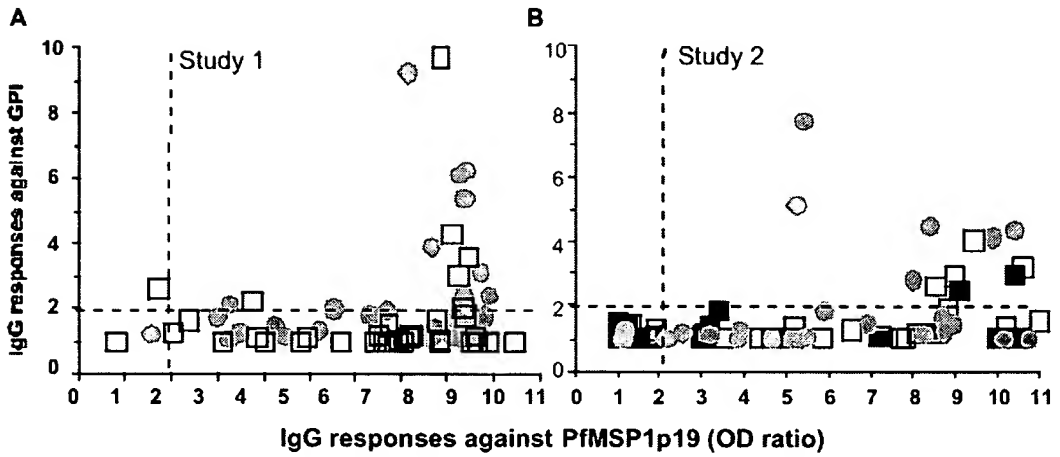

Fig. 2. Distribution of anti-PfMSP1p19 as function of anti-GPI antibody responses in study1 (a) and study2 (b). Shown are scatter plots of anti-PfMSP1p19 (*X*-axis) as function of anti-GPI (*Y*-axis) of hospitalized mild malaria controls (hMM, gray circles) vs. surviving cerebral malaria cases (sCM, open squares). In part b are plotted data from study 2 with mild malaria from health center (MM, gray circles), vs. surviving cerebral malaria cases (sCM, open squares) and fatal cases (fCM, black squares).

3.2. IgG responses to GPI and MSP1p19 in study group 2

The distribution of anti-GPI IgG response for individual cases in CM, MM and NI (uninfected control group) groups is shown in Fig. 1c. The profile of responses in the CM group is similar to that of the CM group in study group 1 (see Fig. 1b), with a restricted number of positives responses leading to similar median values (Table 1). Importantly, there was no significant difference in anti-GPI IgG responses between fatal cases and surviving individuals (CM). The profile of anti-GPI antibody response for MM group is similar to that of anti-GPI antibody response for hMM in study group 1 (see Fig. 1b, c). As in the case of study group 1, the number individuals with positive anti-GPI antibody response are significantly higher in MM group than in CM group (chi square = 4.76; $P = 0.03$). (Fig. 1c).

Importantly, there was no positive response against GPI in the non-infected control group, despite some past infection as indicated by the presence of anti-MSP1 antibodies (Fig. 1d). These results are in agreement with the notion that IgG against GPI are short-lived and boosted by active infection.

In this study, PfMSP1p19 was also strongly recognized by infected individuals (71–87% positive responders), with a non-negligible incidence in urban negative controls (Table 1 and Fig. 1d). Importantly, in the group of hospitalized individuals, a comparison of IgG responses specific for PfMSP1p19 in patients who recovered from cerebral malaria versus those who had fatal outcomes indicated that there was a significantly lower level ($P = 0.012$) of anti-PfMSP1p19 antibody responses (median value of 8.0 and 2.4, respectively) and a significantly lower prevalence of responses (50% vs. 79%, chi square = 5.45; $P = 0.02$).

In addition, as observed in study group 1, there was no relationship between IgG responses against GPIs and MSP1p19 and no age-associated IgG responses despite the inclusion of young individuals in this study (Fig. 2b).

4. Discussion

In this study, we analyzed anti-GPI IgG responses in individuals living in an urban area of low endemicity, who developed cerebral malaria following *P. falciparum* infection. Two groups of patients (CM and MM), considered to be non-immune, were sampled from the same location during different time periods, and categorized on the basis of the clinical outcomes. These individuals, regardless of age and exposure to infective bites, were at risk for clinical episodes, and shared similar observable clinical outcomes of cerebral malaria. Recruitment involved well-documented case–control study, sharing an adequate follow-up and treatments in the intensive care unit of Dakar's hospitals. There was a substantial heterogeneity in such recruitment, as the individual level of medical care and history of infection is partially documented before hospitalization. Inclusion in the study was limited to the transmission season and restricted by the capacity of the intensive care facilities, requiring a cumulative enrollment during two consecutive seasons to collect an adequate number of comparable samples.

The data showed that individuals who developed cerebral malaria had markedly lower levels of anti-GPI antibodies compared to those hospitalized with mild symptoms. This suggests that cerebral malaria is associated with low levels of anti-GPI IgGs, possibly resulting in insufficient anti-GPI neutralizing activity. In contrast, there was no significant difference of anti-PfMSP1p19 antibodies in these two categories of individuals. Interestingly, antibody levels against several *P. falciparum* recombinant conserved antigens other than PfMSP1p19 were similar between hMM and sCM groups (data not shown). This may be related to differences in the kinetics of the production of antibodies against GPI and PfMSP1p19 and other protein antigens. Glycolipids are generally poor antigens compared to proteins and therefore, the anti-GPI antibody response may require repeated exposure,

as compared to rapid boosting of the highly immunogenic MSP1p19 antigen. Even though the anti-GPI antibody responses are considerably lower than those for PfMSP1p19, they possibly influence significantly the development of cerebral malaria.

The presence of comparable levels of anti-PfMSP1p19 antibodies in individuals from both study groups, regardless of disease status, suggests that protective responses against parasite surface protein antigens, which likely control infection by interfering with parasite invasion [17], may not be sufficient for effective control of severe malaria. Thus, the results of our study argue in support of the widely prevailing notion that protective immunity against malaria pathology consists of two major components: "antiparasite" and "antitoxin" immunity [24]. While antibodies against parasite antigens such as PfMSP1p19 can effectively control parasite infection [17,19], and thereby lower the risk of developing severe malaria, anti-GPI antibodies, have been suggested to provide protection against the development of severe symptoms of the disease by neutralizing the activity of GPIs [2]. Although in some cases, depending on host genetic variation, the anti-parasite approach may provide sufficient protection, the combined approach is expected to be much more effective. Thus, the anti-GPI antibody response can be an important and valid target for the development of anti-disease therapies and/or vaccines. However, since anti-GPI antibodies are short-lived, the challenge is to obtain long lasting antibody responses to realize the full potential of GPI-based vaccines [10,12].

An unexpected finding of this study is that, despite elevated mean IgG responses to PfMSP1p19 in all malaria infected groups, there were significantly lower IgG levels in the subgroup of individuals with fatal outcomes to cerebral malaria, compared to those who recovered. These results are consistent with recent findings of a prospective study in Ndiop indicating the requirement for a critical level of anti-PfMSP1p19 antibodies (OD ratio = 7) for a significant association with delayed reinfection following drug cure [19]. In this study, anti-PfMSP1p19 IgG responses in fatal cases were unusually low compared to results of several studies of *P. falciparum* infected individuals (urban and hospital consultants) using the baculovirus expressed PfMSP1p19 antigen (unpublished data). However, specific IgG responses to MSP1p19 do not qualify as prognostic measures for predicting severe disease and/or fatal outcomes in infected individuals, because multiple targets on the merozoite surface are likely to contribute to the prevention of hyper-parasitemia. In addition, since anti-disease immunity is required to achieve complete protection against severe malaria [25], our results show that measures of anti-GPI IgG could not discriminate between recovery and fatal outcomes to cerebral malaria.

In summary, our results argue for a substantial protection-associated role of anti-GPI IgGs against the manifestations of cerebral malaria. However, a direct role for anti-GPI antibodies in neutralizing parasite toxins involved in malaria pathogenesis remains to be demonstrated. Our results call for further confirmation and studies on the mechanisms by which GPIs are able to stimulate host adaptive immune responses to produce anti-GPI antibodies to exploit the potential of this approach for malaria prophylaxis or therapy.

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References

[1] L. Schofield, S. Novakovic, P. Gerold, R.T. Schwarz, M.J. McConville, S.D. Tachado, Glycosylphosphatidylinositol toxin of plasmodium up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and e-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction, J. Immunol. 5 (1996) 1886–1896.

[2] L. Schofield, M.C. Hewitt, K. Evans, M.A. Siomos, P.H. Seeberger, Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria, Nature 6899 (2002) 785–789.

[3] S.D. Tachado, P. Gerold, R. Schwarz, S. Novakovic, M. Mcconville, L. Schofield, Signal transduction in macrophages by glycosylphosphatidylinositols of plasmodium, trypanosoma, and leishmania—activation of protein tyrosine kinases and protein kinase c by inositolglycan and diacylglycerol moieties, Proc. Natl. Acad. Sci. USA 8 (1997) 4022–4027.

[4] D. Channe Gowda, Structure and activity of glycosylphosphatidylinositol anchors of *Plasmodium falciparum*, Microb. Infect. 9 (2002) 983–990.

[5] L. Schofield, F. Hackett, Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites, J. Exp. Med. 1 (1993) 145–153.

[6] R.S. Naik, G. Krishnegowda, D.C. Gowda, Glucosamine inhibits inositol acylation of the glycosylphosphatidylinositol anchors in intraerythrocytic *Plasmodium falciparum*, J. Biol. Chem. 3 (2003) 2036–2042.

[7] M.M. Stevenson, E.M. Riley, Innate immunity to malaria, Nat. Rev. Immunol. 3 (2004) 169–180.

[8] C. Ropert, R.T. Gazzinelli, Signaling of immune system cells by glycosylphosphatidylinositol (GPI) anchor and related structures derived from parasitic protozoa, Curr. Opin. Microbiol. 4 (2000) 395–403.

[9] R.S. Naik, O.H. Branch, A.S. Woods, M. Vijaykumar, D.J. Perkins, B.L. Nahlen, et al., Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis, J. Exp. Med. 11 (2000) 1563–1576.

[10] C.S. Boutlis, D.C. Gowda, R.S. Naik, G.P. Maguire, C.S. Mgone, M.J. Bockarie, et al., Antibodies to *Plasmodium falciparum* glycosylphosphatidylinositols: inverse association with tolerance of parasitemia in Papua New Guinean children and adults, Infect. Immun. 9 (2002) 5052–5057.

[11] J.B. De Souza, J. Todd, G. Krishegowda, D.C. Gowda, D. Kwiatkowski, E.M. Riley, Prevalence and boosting of antibodies to *Plasmodium falciparum* glycosylphosphatidylinositols and evaluation of their association with protection from mild and severe clinical malaria, Infect. Immun. 9 (2002) 5045–5051.

[12] C.S. Boutlis, P.K. Fagan, D.C. Gowda, M. Lagog, C.S. Mgone, M.J. Bockarie, N.M. Anstey, Immunoglobulin G (IgG) responses to *Plasmodium falciparum* glycosylphosphatidylinositols are short-lived and predominantly of the IgG3 subclass, J. Infect. Dis. 10 (2003) 221–229.

[13] S. Diallo, L. Konate, O. Ndir, T. Dieng, Y. Dieng, I. Bah, et al., in: Malaria in the central health district of Dakar (Senegal). Entomological, parasitological and clinical data, Cahier Sante, 187, 2000, pp. 862–865.

[14] A.A. Holder, R.R. Freeman, The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor, J. Exp. Med. 2 (1984) 624–629.

[15] A.F. Egan, J. Morris, G. Barnish, S. Allen, B.M. Greenwood, D.C. Kaslow, et al., Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, pfMSP-1, J. Infect. Dis. 3 (1996) 765–769.

[16] E.M. Riley, S.J. Allen, J.G. Wheeler, M.J. Blackman, S. Bennett, B. Takacs, et al., Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity, Parasite Immunol. 3 (1992) 321–337.

[17] R.A. O'Donnell, T.F. Koning-Ward, R.A. Burt, M. Bockarie, J.C. Reeder, A.F. Cowman, et al., Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria, J. Exp. Med. 12 (2001) 1403–1412.

[18] V. Chitarra, I. Holm, G.A. Bentley, S. Petres, S. Longacre, The crystal structure of C-terminal merozoite surface protein 1 at 1.8 A resolution, a highly protective malaria vaccine candidate, Mol. Cell 4 (1999) 457–464.

[19] R. Perraut, L. Marrama, B. Diouf, D. Fontenille, A. Tall, C. Sokhna, et al., Distinct surrogate markers for protection against *Plasmodium falciparum* infection and clinical malaria identified in a Senegalese community after radical drug cure, J. Infect. Dis. 12 (2003) 1940–1950.

[20] R. Perraut, L. Marrama, B. Diouf, A. Tall, C. Sokhna, J.-F. Trape, et al., Antibodies to the conserved C-terminal domain of the *Plasmodium falciparum* merozoite surface protein-1 and to the merozoite extract and their relationship with in vitro inhibitory antibodies and protection against clinical malaria in a Senegalese village, J. Infect. Dis. 191 (2005) 264–271.

[21] J.F. Trape, Z.E. Lefebvre, F. Legros, G. Ndiaye, H. Bouganali, P. Druilhe, et al., Vector density gradients and the epidemiology of urban malaria in Dakar, Senegal, Am. J. Trop. Med. Hyg. 2 (1992) 181–189.

[22] J.C. Pizarro, V. Chitarra, D. Verger, I. Holm, S. Petres, S. Dartevelle, et al., Crystal structure of a Fab complex formed with PfMSP1-19, the C-terminal fragment of merozoite surface protein 1 from *Plasmodium falciparum*: a malaria vaccine candidate, J. Mol. Biol. 5 (2003) 1091–1103.

[23] R. Perraut, M. Guillotte, I. Drame, B. Diouf, J.-F. Molez, A. Tall, et al., Evaluation of anti-*Plasmodium falciparum* antibodies in Senegalese adults using different types of crude extracts from various strains of parasite, Microb. Infect. 1 (2002) 31–35.

[24] J.H. Playfair, J. Taverne, C.A. Bate, J.B. De Souza, The malaria vaccine: anti-parasite or anti-disease? Immunol. Today 1 (1990) 25–27.

[25] N.P. Day, T.T. Hien, T. Schollaardt, P.P. Loc, L.V. Chuong, T.T. Chau, et al., The prognostic and pathophysiologic role of pro- and anti-inflammatory cytokines in severe malaria, J. Infect. Dis. 4 (1999) 1288–1297.

tion in promoting T cell proliferation in response to TCR and IL-2 activation. IL-2 receptor expression is not altered, and the T cells are able to produce cytokines in these STAT5A/B double knockout mice (*18*). It is possible that the TCR needs to directly activate STAT5 to induce some expression of immediate early genes, such as the CD69 gene, which has a role in T cell proliferation (*19*). Thus, our results suggest a route of TCR signaling in which STAT5 is directly activated and may contribute to early gene regulation and T cell proliferation.

References and Notes

1. A. Weiss and D. R. Littman, *Cell* **76**, 263 (1994); C. A. Janeway Jr. and K. Bottomly, *Semin. Immunol.* **8**, 108 (1996).
2. T. J. Molina *et al.*, *Nature* **357**, 161 (1992); D. B. Straus and A. Weiss, *Cell* **70**, 585 (1992); L. Karnitz *et al.*, *Mol. Cell. Biol.* **12**, 4521 (1992).
3. M. Reth, *Annu. Rev. Immunol.* **10**, 97 (1992); L. E. Samelson and R. D. Klausner, *J. Biol. Chem.* **267**, 24913 (1992); L. R. Howe and A. Weiss, *Trends Biochem. Sci.* **20**, 59 (1995); R. M. Perlmutter, *Cancer Surv.* **22**, 85 (1995).
4. G. R. Crabtree, *Science* **243**, 355 (1989); A. Rao, C. Luo, P. G. Hogan, *Annu. Rev. Immunol.* **15**, 707 (1997).
5. J. E. Darnell Jr., I. M. Kerr, G. R. Stark, *Science* **264**, 1415 (1994); X. Y. Fu, *J. Leukocyte Biol.* **57**, 529 (1995); J. N. Ihle *et al.*, *Annu. Rev. Immunol.* **13**, 369 (1995); W. J. Leonard and J. J. O'Shea, *ibid.* **16**, 293 (1998).
6. X. Y. Fu and J. J. Zhang, *Cell* **74**, 1135 (1993); H. B. Sadowski, K. Shuai, J. E. Darnell Jr., M. Z. Gilman, *Science* **261**, 1739 (1993); F. W. Quelle *et al.*, *J. Biol. Chem.* **270**, 20775 (1995); C.-L. Yu *et al.*, *Science* **269**, 81 (1995); X. Cao, A. Tay, G. R. Guy, Y. H. Tan, *Mol. Cell. Biol.* **16**, 1595 (1996); D. W. Leaman *et al.*, *ibid.*, p. 369; Y. E. Chin *et al.*, *Science* **272**, 719 (1996).
7. H. Nakajima *et al.*, *Immunity* **7**, 691 (1997).
8. C. A. Janeway Jr. *et al.*, *Immunol. Rev.* **101**, 39 (1988); T. Kupper, M. Horowitz, F. Lee, R. Robb, P. M. Flood, *J. Immunol.* **138**, 4280 (1987).
9. The Th2 clone D10 cells were maintained in Click's medium supplemented with fetal bovine serum (5%) and stimulated with B10BR splenocytes and hen egg conalbumin (0.1 mg/ml) (Sigma) every 3 to 4 weeks. For TCR cross linking, cells were kept on ice for 15 min, incubated with clonotypic antibody 3D3 to D10 TCR (5 μg/ml) on ice for an additional 15 min, washed, and treated with anti–mouse immunoglobulin G (Southern Biotechnology, Birmingham, AL; 8 μg/ml) at 37°C. Phospho-STAT5 analysis: Cells were collected by centrifugation and immediately lysed by boiling in electrophoresis loading buffer. Phosphorylation of STAT5 was detected by protein immunoblotting with specific antibody to phosphotyrosine 694 of STAT5 (New England Biolabs, Beverly, MA; Zymed, San Francisco, CA). IL-2 was obtained from Chiron (Emeryville, CA).
10. T. Welte and X.-Y. Fu, unpublished results.
11. 293T cells were transiently transfected with DNA by calcium phosphate precipitation. Experiments were carried out 2 days after transfection. Electrophoretic mobility-shift assay (EMSA) was done with whole-cell lysates that were incubated with nonspecific competitor DNA and ^{32}P-labeled β-casein SIE (5′-TGTGGACTTCTTGGAATTAAGGGACTTTTG-3′) or CD69-SIE (5′-GATCCGATTCCTGGAAAATG-3′). Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.5× tris-borate EDTA.
12. B. K. al-Ramadi, T. Nakamura, D. Leitenberg, A. L. Bothwell, *J. Immunol.* **157**, 4751 (1996).
13. Immunoprecipitation: 293T cell lysates were prepared in a lysis buffer containing Digitonin as detergent [4 × 10^6 cells, 250 μl of 1% Digitonin, 20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, aprotinin (10 mg/ml), leupeptin (10 mg/ml), pepstatin (1 mg/ml), and 250 μM o-Vanadate]. Lysates were incubated with anti-STAT5A (1.2 μg per sample; Santa Cruz Biotechnology, Santa Cruz, CA), prebound to protein A agarose beads, for 3 hours and washed two times in 1% Digitonin lysis buffer and two times in 0.1% Digitonin lysis buffer. T cell clone cells and primary T cells were lysed in a lysis buffer containing 1% Brij instead of Digitonin and supplemented with 10 mM $Na_4P_2O_7$ (10^7 cells, 250 μl). Anti-STAT5 and anti–CD3-ε (145-2C11) immunoprecipitations, agarose-conjugated anti-STAT5B, and anti–NF-κB (7.5 μg per sample) were added for 2 hours followed by washing steps. Protein immunoblots were done with anti-STAT5 (Santa Cruz Biotechnology), anti-phosphotyrosine (4G10), anti–TCR-α, anti–TCR-ζ, and anti-Lck (Santa Cruz Biotechnology).
14. D. Leitenberg, Y. Boutin, S. Constant, K. Bottomly, *J. Immunol.* **161**, 1194 (1998).
15. FLAG-tagged STAT5 was obtained by replacing the NH_2-terminal methionine of mouse STAT5A by FLAG-encoding sequence. In FLAG STAT5 Y694F, tyrosine (Y) 694 was changed to phenylalanine (F) (QuikChange Site-Directed Mutagenesis Kit; Stratagene). Expression of FLAG-tagged proteins was detected with antibody M2 (Kodak). Stable transfection of D10 cells was done as described (*12*). Linearized plasmid DNA (20 μg) was transfected by electroporation of antigen-stimulated D10 cells (2 × 10^7), followed by selection in a medium containing G418 (1.4 mg/ml) (Gibco-BRL). Stable clones were obtained about 1 1/2 months later, restimulated every 2 weeks, and expanded in the presence of IL-2 (3 U/ml).
16. Proliferation assay: D10 cells and transfectants (2.5 × 10^4) were incubated with mitomycin C–treated splenocytes of B10.BR mice (5 × 10^4) and the agonistic peptide CA134-146. After 60 hours, 1 μCi of [^{3}H]thymidine was added for 4 hours, followed by harvest and liquid scintillation counting.
17. C. Beadling *et al.*, *EMBO J.* **13**, 5605 (1994).
18. S. Teglund *et al.*, *Cell* **93**, 841 (1998).
19. W. M. Yokoyama, S. R. Maxfield, E. M. Shevach, *Immunol. Rev.* **109**, 153 (1989); S. F. Ziegler *et al.*, *J. Immunol.* **152**, 1228 (1994).

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CD1d-Restricted Immunoglobulin G Formation to GPI-Anchored Antigens Mediated by NKT Cells

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Immunoglobulin G (IgG) responses require major histocompatibility complex (MHC)–restricted recognition of peptide fragments by conventional $CD4^+$ helper T cells. Immunoglobulin G responses to glycosylphosphatidylinositol (GPI)-anchored protein antigens, however, were found to be regulated in part through CD1d-restricted recognition of the GPI moiety by thymus-dependent, interleukin-4–producing $CD4^+$, natural killer cell antigen 1.1 [$(NK1.1)^+$] helper T cells. The CD1-NKT cell pathway regulated immunogobulin G responses to the GPI-anchored surface antigens of *Plasmodium* and *Trypanosoma* and may be a general mechanism for rapid, MHC-unrestricted antibody responses to diverse pathogens.

NKT cells are unusual $CD4^+$, $NK1.1^+$ lymphocytes (*1*) that produce interleukin-4 (IL-4) rapidly in response to T cell receptor (TCR) ligation (*2*). These cells have a skewed $V_\alpha V_\beta$ TCR repertoire ($V_\alpha 14$ and $V_\beta 8$ in mice) (*3*), suggesting that they are positively selected by a limited range of ligands. Murine NKT cells are positively selected by cortical thymocytes expressing the non-MHC–encoded but MHC class I–like molecule CD1d (*1*). The related human CD1b and CD1c molecules can elicit cytolytic and interferon-γ responses by presenting mycobacterial glycolipid antigens to $CD8^+$ or $CD4^-CD8^-$ T cells (*4*). Murine $V_\alpha 14^+$ NKT cells recognize synthetic α-galactosylceramide in the context of CD1d (*5*), and murine CD1d in transfected human T2 cells associates with phosphatidylinositol (PI)-containing compounds that may be GPIs (*6*). Therefore, $CD4^+$ NKT cells

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may participate in CD1d-restricted recognition of lipid antigens. However, the natural ligand and functional significance of NKT cells in immune responses in vivo remains unclear. The present study was initiated to test the assumption of MHC restriction of immunoglobulin G (IgG) responses to the GPI-anchored surface antigens of important protozoal pathogens. We have found instead that several such responses are controlled in part by CD1d-restricted recognition of GPI moieties by $CD4^+$ NKT cells.

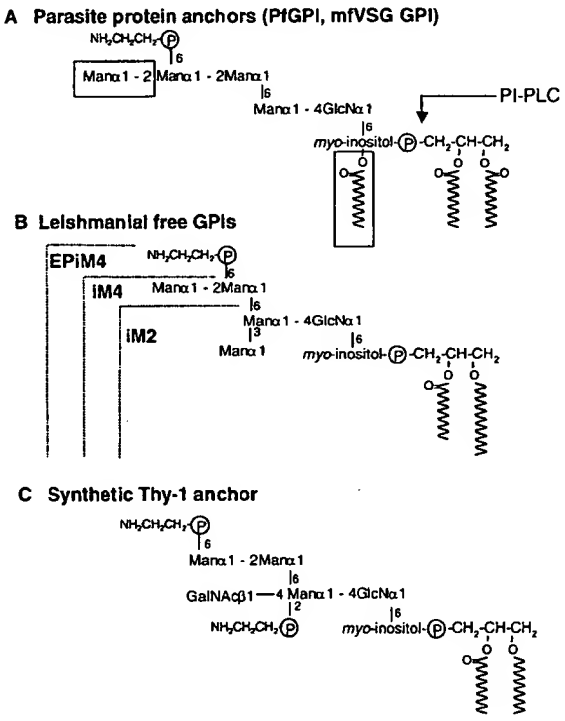

Fig. 1. Diagrammatical representation of GPI structures used in this study. Purification and compositional analyses are as described (*12–14*). (**A**) COOH-terminal GPIs from *T. brucei* and *P. falciparum*. Boxed areas represent modifications found in PfGPI. The cleavage site of mfVSG by phosphatidylinositol-specific phospholipase C (PI-PLC) is indicated. (**B**) Free iM2, iM4, and EP-iM4 GPIs of *L. mexicana*. Nomenclature is as described (*14*), where all isomers contain one mannose in α1-3 linkage, EP indicates ethanolamine phosphate, and M2 and M4 indicate the number of mannose residues, as shown. (**C**) Chemically synthesized rat brain Thy-1 GPI.

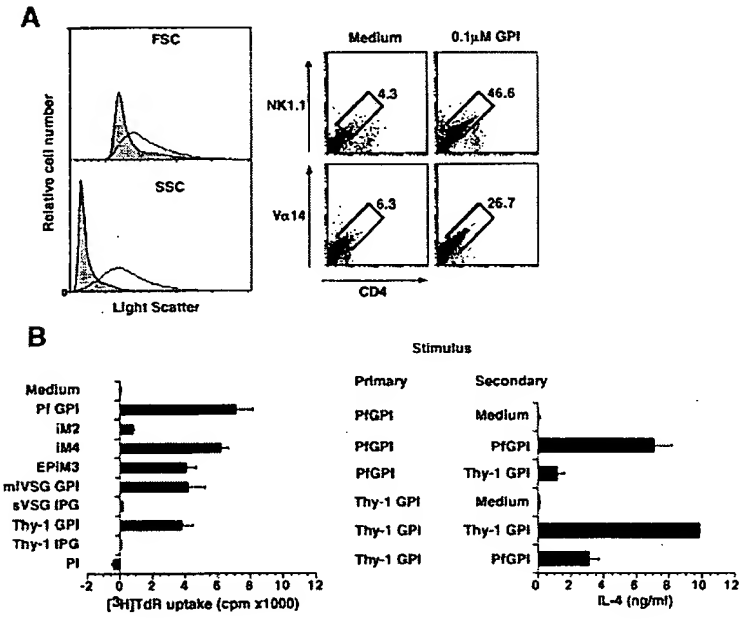

Fig. 2. Response of peripheral NKT cells to purified GPIs in vitro. (**A**) As determined by forward (FSC) and side (SSC) light scatter, splenocytes from SPZ-primed class $II^{-/-}$ donors proliferate within 48 hours exposure to PfGPI (unshaded) compared with medium controls (shaded). The responding cells are $NK1.1^+$, $CD4^+$ and include a $V_\alpha14^+$, $CD4^+$ subset (*25*). (**B**) Splenocytes from class $II^{-/-}$ donors were exposed to various antigens, and [^{3}H]TdR incorporation was determined after 4 days. Other cultures were exposed to PfGPI or Thy-1 GPI for 4 days, washed, and cultured in IL-2 (10 U/ml) for 2 days, followed by replating with irradiated wild-type APCs and restimulation with either PfGPI or Thy-1 GPI for 48 hours. IL-4 levels in the supernatant were determined by capture ELISA.

T cell–dependent IgG responses to protein antigens are thought to be exclusively MHC class II–restricted. However, allogeneic bone-marrow irradiation chimeras were similar to syngeneic controls in responding to malaria sporozoites (SPZs) with IgG to the circumsporozoite (CS) protein, despite being unable to respond to the nominal protein antigens tetanus toxoid (TT) or a full-length recombinant *Plasmodium falciparum* CS protein (recCS) (*7*). Nude mice cannot respond to SPZs with anti-CS IgG, and passive transfer of depleting antibodies to CD4 into euthymic animals abolishes the anti-CS response to SPZs (*8*). However, nude mice engrafted with irradiated neonatal allogeneic thymi mounted anti-CS IgG responses to SPZs similar to those of recipients of syngeneic thymi, but did not respond to recCS or TT (*7*). Mice lacking both class II and class II–restricted $CD4^+$ T cells, and that are unable to respond to T-dependent antigens (*9*), produced anti-CS IgG (mean $\log_2$ reciprocal titer of 10) in response to SPZs (*10*). Thus, $CD4^+$ T cells are required for the IgG response to the native CS protein, but this may proceed through a MHC class II–independent route.

The native CS protein is posttranslationally modified by a GPI anchor (*11*), whereas TT and recCS are not. To determine whether the GPI anchor accounts for the difference in immunological behavior of the proteins, we purified COOH-terminal GPIs from affinity-purified GPI-anchored proteins of *P. falciparum* (PfGPI) and *Trypanosoma brucei* membrane-form variant surface glycoprotein (mfVSG) (*12*) (Fig. 1A), and nonprotein-linked free GPIs from *Leishmania mexicana* (*12*, *13*) (Fig. 1B). The compositional purity of these latter molecules was confirmed by gas chromatography–mass spectrometry (GC-MS). In addition, a phosphorylated and lipidated mammalian GPI based on the rat brain Thy-1 GPI (Fig. 1C), and the corresponding inositolphosphoglycan (IPG) lacking a lipid tail, both chemically synthesized by *n*-pentenyl glucoside strategy and compositionally pure by ^{1}H nuclear magnetic resonance (NMR) analysis (*14*), were also used. To generate responses to the hapten fluorescein (FLU), native and synthetic GPIs and IPG were exposed to 2-iminothiolane to introduce a sulfhydryl onto free amino groups, desalted, and conjugated in a molar ratio of 1:1 to fluoresceinated, maleimide-activated ovalbumin (OVA^{FLU}). In contrast to sham-OVA^{FLU} alone or sham-OVA^{FLU} mixed with equal molar amounts of free PfGPI, PfGPI-OVA^{FLU} conjugates were able to induce anti-FLU IgG1 formation in MHC class $II^{-/-}$ mice (mean $\log_2$ reciprocal titer of 8). Similar IgG responses

were obtained in class $II^{-/-}$ mice with the mfVSG of *T. brucei*, but not the deacylated soluble VSG derived by PI-specific phospholipase C (PI-PLC) hydrolysis (Fig. 1A), demonstrating that the GPI lipid domain is required, and the GPI glycan is not sufficient, for the phenomenon. This was confirmed by comparing responses to OVA^{FLU} conjugated to either synthetic Thy-1 GPI or Thy-1 IPG lacking fatty acid (log_2 reciprocal titer of 9.75 versus no response, respectively). Thus, IgG responses in class $II^{-/-}$ mice require linkage of antigen to GPI with an intact lipid, which may be composed of diacylglycerol or alkylacylglycerol.

Although lacking conventional T cells, class $II^{-/-}$ mice retain a diverse population of MHC-nonrestricted $CD4^+$ α/β TCR^+ T cells, including NKT cells and other $CD4^+$ cells selected on CD1 (*9*, *15*). We therefore hypothesized that IgG responses to native and synthetically conjugated GPI-anchored proteins in both wild-type and class $II^{-/-}$ mice proceed from CD1d-restricted presentation of GPIs to nonconventional T cells. To test this hypothesis, we examined the in vitro proliferative and cytokine responses to purified malarial GPI of splenocytes from animals primed with malaria SPZs. There was a marked increase in both the relative and absolute numbers of $NK1.1^+$ $CD4^+$ blastoid cells responding to GPI from both class $II^{-/-}$ (Fig. 2A) and wild-type mice. A high frequency of both $V_\alpha14^+$ $CD4^+$ (Fig. 2A) and $V_\beta8^+$ cells was also detected in the responding population. No exogenous cytokines were required for this proliferation, but supplementation of cultures with IL-2 (5 U/ml) increased the level of response.

To examine the fine specificity of responding cells, we exposed splenocytes from wild-type and class $II^{-/-}$ animals primed to *P. berghei* SPZs to 0.5 μM of the GPI structures shown in Fig. 1, together with dipalmitoyl-PI. The cells responded to a similar degree to most intact GPIs, but only weakly to the iM2 GPI with truncated glycan and not at all to glycans lacking the fatty acid domain, or to PI (Fig. 2B). Thus, both glycan and fatty acids are required for recognition, and NKT cells from SPZ-primed donors respond to a range of GPIs from diverse protozoal and mammalian taxa. However, because the full range of structures presented to the host under these priming conditions is not known, the results may reflect either broad recognition of diverse antigens by the general population of NKT cells or heterogeneous responses from a clonally mixed population. To further distinguish between these possibilities, cells were expanded in the presence of either PfGPI or Thy-1 GPI for 4 days, rested in IL-2, then restimulated with homologous or heterologous antigen. Cells expanded by either antigen responded significantly less well to the heterologous stimulus (Fig. 2B). Analysis of antigen-specific frequencies after in vitro culture revealed no increase above background levels in response to PfGPI in naïve donors, but a clear increase in antigen-reactive NKT cells from SPZ-primed donors (7 to 30% of total) (*16*). However, naïve and primed donors both mounted significant responses to the iM4 GPI (to 5% of NKT cells) (*16*). Together, the data are consistent with the reported fine specificity of CD1-restricted $CD4^-CD8^-$ human T cells for the glycan component of glycolipids (*4*, *17*), suggesting that antigen priming expands clonally diverse NKT cells that are able to discriminate among structurally distinct GPIs, but that high precursor frequencies for some GPIs may occur even among naïve animals kept under specific-pathogen–free conditions.

Cells that have been positively selected on NK1.1 and CD4 proliferate and produce cytokines specifically in response to TCR-mediated signals (*2*, *18*). When sorted $NK1.1^+$ $CD4^+$ cells from wild-type and class $II^{-/-}$ mice that had been primed to SPZs were cultured with irradiated wild-type or class $II^{-/-}$ antigen-presenting cells (APCs), they responded to purified GPIs, as determined by incorporation of [^{3}H]thymidine ([^{3}H]TdR) and the production of high levels of IL-4 (Fig. 3A). No proliferation in the absence of APCs indicated that GPIs do not provide a direct activation signal to NKT cells that is sufficient to induce cell growth. NKT cells did not respond to GPIs when cultured with irradiated APCs from β_2-microglobulin$^{-/-}$ ($\beta_2M^{-/-}$) and CD1.1/CD1.$2^{-/-}$ ($CD1^{-/-}$) (*19*) donors, or with wild-type and class $II^{-/-}$ APCs in the presence of anti-CD1.1 (1B1) (*20*), but responded fully in the presence of isotype controls (Fig. 3A). The proliferative and IL-4 response to PfGPI of NKT cells and the $V_\alpha14^+$, $CD4^+$ subset in unfractionated splenocytes could also be blocked by the anti-CD1 monoclonal antibody 1B1 (Fig. 3B). Thus, the recognition of GPIs by NKT cells is MHC-independent and CD1-restricted. In addition, NKT cells produced IL-4 in response to CD1.1-transfected J774 macrophages (*20*) in the absence of exogenous antigen, but not in response to sham-transfected controls. Nonetheless, the response was enhanced when CD1.1-transfectants were pulsed with PfGPI (Fig. 3A). The response of NKT cells to CD1.1, observed previously (*5*, *18*), has been adduced in support of the proposition that this cell population may play a physiological role in the absence of associative recognition of antigen (*5*, *21*). However, as reported (*5*, *18*), in the absence of exogenous antigen no cytokine expression was detected in response to APCs expressing normal levels of CD1.1. Thus, high levels of CD1.1 expression in transfected cells may alone be sufficient to drive proliferation. Alternatively, because NKT cells can respond to mammalian GPIs [for example, syn-

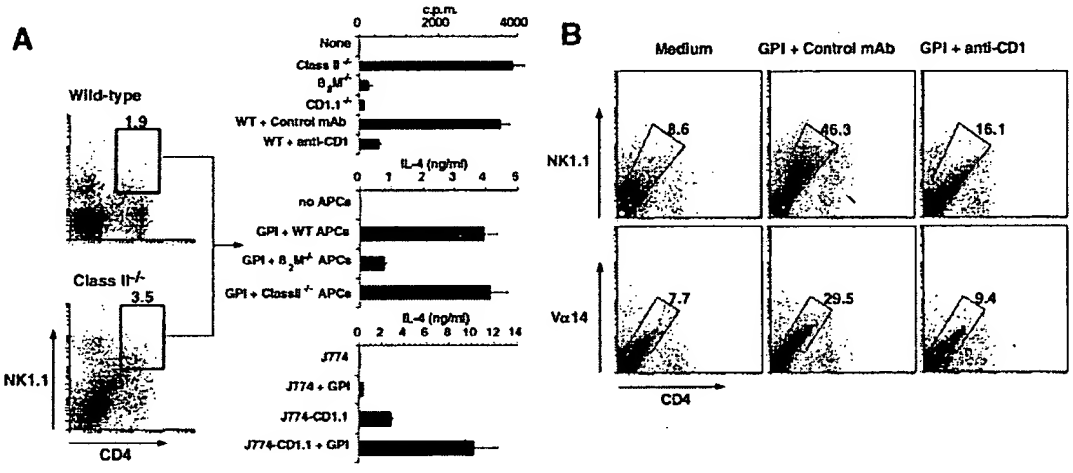

Fig. 3. The proliferative and IL-4 response of NKT cells to PfGPI is MHC-independent and CD1-restricted. (**A**) Sorted $NK1.1^+$, $CD4^+$ cells (2×10^4) from wild-type or class $II^{-/-}$ donors were placed in triplicate with or without purified GPI on irradiated splenocyte APCs from wild-type (WT), class $II^{-/-}$, $\beta_2M^{-/-}$, or $CD1^{-/-}$ donors, or CD1.1-transfected and sham-transfected J774 macrophages. [^{3}H]TdR incorporation was determined after 3 days, or IL-4 production in the presence or absence of anti-CD1 was determined as in Fig. 2. (**B**) Splenocytes from SPZ-primed class $II^{-/-}$ donors were exposed to PfGPI in the presence or absence of anti-CD1 or isotype control and taken for flow cytometric analysis after 3 days.

thetic Thy-1 GPI (Fig. 2B)], CD1.1-transfectants may be able to present endogenous GPIs to NKT cells, as suggested by the association of CD1d in transfected T2 cells with PI-containing compounds (*5*). Thus, CD1 may not be "empty," and self-reactive NKT cells may arise through incomplete negative selection. Such a possibility may explain the nonantigen-specific regulatory activity of NKT cells (*2*, *22*).

NKT cells can induce Ig class switch in B cells exposed to anti-IgD (*2*). Extending to antigen-specific systems, NKT cells cooperated with B cells by ELISPOT assay in CD1-restricted IgG formation to GPI-OVA^{FLU} and native *P. berghei* CS protein formation, but not to OVA^{FLU} (Fig. 4A). To determine, therefore, whether CD1.1- or CD1.2-restricted antibody formation was a major or minor contributor to the IgG response to GPI-anchored proteins and SPZs in vivo, we exposed CD1$^{-/-}$ mice and wild-type controls to mfVSG^{FLU}, SPZs, or recCS. Responses to mfVSG^{FLU} and SPZs were significantly curtailed in CD1$^{-/-}$ mice (Fig. 4B), indicating that under these conditions the CD1-restricted pathway of IgG formation is an important component of responses to the native CS protein. Both groups responded equally to recCS, confirming that class II–dependent responses are unaffected by loss of CD1. It is not yet clear whether the IgG responses to SPZs in CD1$^{-/-}$ mice result from class II–restricted responses to the intact GPI-anchored CS protein or to a proportion of the antigen adventitiously deacylated in these preparations, or from a non–class II, non-CD1 pathway.

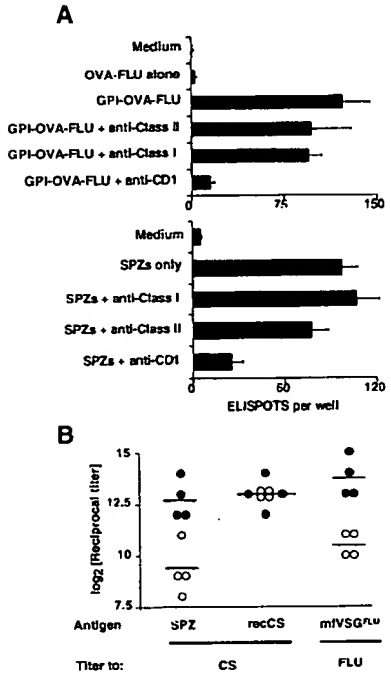

Fig. 4. CD1-restricted antibody formation to neo-GPI-proteins and malaria SPZs. (**A**) Donor *nu/nu* mice were primed twice with *P. berghei* SPZs or twice with LPS^{FLU}. Splenocytes were cultured in the presence of IL-2 (10 U/ml), with and without antigen (0.1 μg/ml sham-OVA^{FLU}, PfGPI-OVA^{FLU}, or 5 × 10^4 SPZs), anti-class I, anti-class II, and anti-CD1, with 10^4 NKT cells from SPZ-primed class II$^{-/-}$ donors. Antigen-specific IgG production was quantified by ELISPOT against fluoresceinated dog serum albumin for responses to OVA^{FLU}, and rCS for responses to SPZs. (**B**) Responses of CD1.1/CD1.2$^{-/-}$ (○) and Balb/c wild-type mice (●) to SPZs, recCS, and mfVSG^{FLU}. IgG titers were determined as described (*7*).

GPIs are widespread among eukaryotes, and the expression of GPI-anchored proteins and free GPIs is particularly abundant among the parasitic protozoa. Because CD1-restricted NKT cells can recognize GPIs from diverse taxa (Fig. 2B), CD1-restricted IgG formation may represent a general mechanism for rapid responses to the GPI-anchored surface antigens of various pathogens. The native CS protein appears to be more immunogenic than the recombinant version. Inoculation of as few as 10^4 nonreplicating irradiated SPZs (1 ng of native CS protein, assuming 10^6 copies per cell) is sufficient to elicit an antibody response comparable to that obtained with 1 to 10 μg of recCS. The rapid responses of NKT cells in vivo (*2*) and a relatively high precursor frequency of antigen-specific NKT cells may contribute to this phenomenon. Thus, consistent with the "danger model" of pathogen-initiated immune responses (*23*), CD1-restricted immunity may be intermediate between the innate "pattern recognition" and adaptive immune systems. MHC-restricted nonresponsiveness to malarial surface antigens has been proposed to be a major obstacle to the development of vaccines (*24*). Because both human and murine CD1 molecules are relatively nonpolymorphic, GPI anchors may provide universal T cell sites, overcoming MHC restriction in antibody responses to various pathogens.

References and Notes

1. H. Arase, N. Arase, K. Ogasawara, R. A. Good, K. Onoe, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6506 (1992); A. Bendelac, N. Killeen, D. R. Littman, R. H. Schwartz, *Science* **263**, 1774 (1994); A. Bendelac, *J. Exp. Med.* **182**, 2091 (1995).
2. T. Yoshimoto and W. E. Paul, *J. Exp. Med.* **179**, 1285 (1994); T. Yoshimoto, A. Bendelac, C. Watson, J. Hu-Li, W. E. Paul, *Science* **270**, 1845 (1995); T. Yoshimoto, A. Bendelac, J. Hu-Li, W. E. Paul, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11931 (1995).
3. M. Bix, M. Coles, D. Raulet, *J. Exp. Med.* **178**, 901 (1993); O. Lantz and A. Bendelac, *J. ibid.* **180**, 1097 (1994); Y. Makino, R. Kanno, T. Ito, K. Higashino, M. Taniguchi, *Int. Immunol.* **7**, 1157 (1995).
4. S. Porcelli, C. T. Morita, M. B. Brenner, *Nature* **360**, 593 (1992); E. M. Beckman *et al.*, *ibid.* **372**, 691 (1994); P. A. Sieling *et al.*, *Science* **269**, 227 (1995).
5. T. Kawano *et al.*, *Science* **278**, 1626 (1997); L. Brossay *et al.*, *J. Immunol.* **161**, 5124 (1998).
6. S. Joyce *et al.*, *ibid.* **279**, 1541 (1998).
7. Irradiated H-2 congenic mice received 10^7 T cell–depleted syngeneic or allogeneic bone-marrow cells. Thymic lobes from neonatal Balb/c, Balb/B, and Balb/K mice were irradiated and implanted into adult Balb/c *nu/nu* mice. Mice were rested 12 weeks before testing for chimerism. *P. falciparum* and *P. berghei* SPZs were dissected from *Anopheles freeborni* and *A. stephensi*, respectively. Some preparations were kept at −70°C before use. Mice were primed with TT, recCS, or SPZs, boosted after 1 week, and IgG responses analyzed by enzyme-linked immunosorbent assay (ELISA). RecCS, derived from the T4 strain and consisting of the entire sequence except for the COOH-terminal 21 amino acids (GPI-anchor signal sequence), was used as immunizing antigen and to detect responses to *P. falciparum* SPZs. The *P. berghei* rCS (detection antigen only for *P. berghei* SPZs) encompasses amino acids 81 to 277 (*26*). Antigen-specific end titers were defined as the last titration giving values statistically different from binding to plates without antigen. A $\log_2$ reciprocal titer of 4.0 was the background cutoff. To TT, recCS, and *P. berghei* SPZs, syngeneic thymic chimeras mounted mean $\log_2$ reciprocal IgG titers of 15.14, 13.5, and 13.5, respectively. The equivalent values for allogeneic chimeras were 4.5, 4.25, and 13. For syngeneic bone-marrow chimeras the equivalent values were 15.11, 11.25, and 14.6, and for allogeneic chimeras 8.5, 7.25, and 15.25, respectively.
8. W. R. Weiss, M. Sedegah, J. A. Berzofsky, S. L. Hoffman, *J. Immunol.* **151**, 2690 (1993).
9. D. Cosgrove *et al.*, *Cell* **66**, 1051 (1991); M. J. Grusby, R. S. Johnson, V. E. Papaioannou, L. H. Glimcher, *Science* **253**, 1417 (1991); F. Köntgen, G. Süss, C. Stewart, M. Steinmetz, H. Bluethmann, *Int. Immunol.* **5**, 957 (1993).
10. Immunglobulin G responses to rCS, recCS, or FLU were determined as in (*7*) in class II$^{-/-}$ and wild-type mice receiving *P. berghei* SPZs, recCS, mfVSG^{FLU}, sVSG^{FLU}, or OVA^{FLU}, either sham-conjugated (sham-OVA^{FLU}) or conjugated to *P. falciparum* GPI (PfGPI-OVA^{FLU}), Thy-1 GPI (Thy-1-OVA^{FLU}), or Thy-1 IPG (IPG-OVA^{FLU}). Unlike the wild-types, class II$^{-/-}$ animals failed to raise IgG to recCS, sVSG^{FLU}, sham-OVA^{FLU}, and IPG-OVA^{FLU} (mean $\log_2$ reciprocal titers <4). However, they produced IgG in response to SPZs, PfGPI-OVA^{FLU}, mfVSG^{FLU}, and Thy-1-OVA^{FLU} ($\log_2$ reciprocal titer of 10, 8, 8.25, and 9.75, respectively). Immunoglobulin isotypes included IgG1, IgG2a, and IgG2b.
11. P. Moran and I. W. Caras, *J. Cell Biol.* **125**, 333 (1994).
12. mfVSG and GPI-anchored *P. falciparum* proteins, purified by high-performance liquid chromatography and affinity chromatography (*27*), were extracted in chloroform/methanol (2:1), precipitated with acetone, solubilized and reduced, diluted in 5 mM $CaCl_2$, digested for 72 hours at 37°C with Pronase B, purified over Octyl-Sepharose, partitioned between water and water-saturated butanol (PfGPI), and purified by high-performance thin-layer chromatography (HPTLC) (R_f = 0.05) with chloroform/methanol/acetic acid/water (C/M/HAc/W; 25:15:4:2). CMW (1:2:0.8) extracts of *L. mexicana* promastigotes were purified over Octyl-Sepharose followed by HPTLC with a solution of CM and 1 N NH_4OH (10:10:3). The compositional purity of the latter molecules was confirmed by GC-MS (*13*) after acid methanolysis and trimethylsilyl (TMS) derivatization for neutral monosaccharide analysis, and acid hydrolysis (6N HCl, 110°C, 16 hours) and TMS derivatization, with selected ion monitoring for mass-to-charge ratio (*m/z*) of 305 and 318. *scyllo*-Inositol was used as internal standard throughout. Compositional molar ratios were as predicted, and no contaminating carbohydrates or sugars were detected. The specific values are published elsewhere (*13*), as are the ^{1}H NMR compositional analyses demonstrating purity of the synthetic Thy-1 GPI and IPG (*14*).
13. M. J. McConville and J. M. Blackwell, *J. Biol. Chem.* **266**, 15170 (1991); M. J. McConville, T. A. C. Collidge, M. A. J. Ferguson, P. Schneider, *ibid.* **268**, 15595 (1993); J. E. Ralton and M. J. McConville, *ibid.* **273**, 4245 (1998).
14. A. S. Campbell and B. Fraser-Reid, *J. Am. Chem. Soc.* **117**, 10387 (1995).

15. A. Bendelac *et al.*, *Science* **268**, 863 (1995); S. Cardell *et al.*, *J. Exp. Med.* **182**, 993 (1995).
16. Splenocytes from naïve and SPZ-primed class II$^{-/-}$ donors were bulk cultured in flat-bottomed plates in the presence or absence of PfGPI and iM4 for 4 days. The proportion of NKT cells expressing intracellular IL-4 was measured by flow cytometry after subtraction of background staining by isotype-matched fluorescein isothiocyanate–conjugated control monoclonal antibody R35-38.
17. D. B. Moody, *et al.*, *Science* **278**, 283 (1997).
18. H. Chen and W. E. Paul, *J. Immunol.* **159**, 2240 (1997); H. Chen, H. Huang, W. E. Paul, *ibid.* **158**, 5112 (1997).
19. S. T. Smiley, M. H. Kaplan, M. J. Grusby, *Science* **275**, 977 (1997).
20. L. Brossay *et al.*, *J. Immunol.* **159**, 1216 (1997); L. Brossay *et al.*, *ibid.* **160**, 3681 (1998).
21. S. H. Park, J. H. Roark, A. Bendelac, *ibid.*, p. 3128.
22. S. A. Porcelli, *Adv. Immunol.* **59**, 1 (1995); A. Bendelac, M. N. Rivera, S.-H. Park, J. H. Roark, *Annu. Rev. Immunol.* **15**, 535 (1997).
23. P. Matzinger, *Annu. Rev. Immunol.* **12**, 991 (1994).
24. M. F. Good, S. Kumar, L. H. Miller, *Immunol. Today* **9**, 351 (1988).
25. Monoclonal antibodies were to CD1.1 (1B1), CD4 (H129.19), CD16 (2.4G2), NK1.1 (PK136), V_β8.1/8.2 (KJ16), V_α14 (CMS-1), I-A^b (M5/114), H-2K^b/H2-D^b (28-8-6), H-2 (M1/42), and IL-4 (11B11, BVD6-24G2, BVD4-1D11).
26. P. J. Romero *et al.*, *Eur. J. Immunol.* **18**, 1951 (1988).
27. M. W. Clarke, R. W. Olafson, T. W. Pearson, *Mol. Biochem. Parasitol.* **17**, 19 (1985); L. Schofield and F. Hackett, *J. Exp. Med.* **177**, 145 (1993); S. D. Tachado *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4022 (1997).
28. We thank M. Kronenberg for the CD1-transfectants and monoclonal antibody 1B1 and M. Taniguchi for CMS-1. Supported by the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, the Australian Academy of Science (L.S.), NIH grant GM 41071 (B.F.-R. and S.C.), INSMED (B.F.-R.), NIH grant AI-40171, and a gift from the Mathers Foundation (M.J.G.). M.J.M. is a Wellcome Trust Senior Research Fellow. M.J.G. is a Scholar of the Leukemia Society of America.

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Grassland Vegetation Changes and Nocturnal Global Warming

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Global minimum temperatures (T_{MIN}) are increasing faster than maximum temperatures, but the ecological consequences of this are largely unexplored. Long-term data sets from the shortgrass steppe were used to identify correlations between T_{MIN} and several vegetation variables. This ecosystem is potentially sensitive to increases in T_{MIN}. Most notably, increased spring T_{MIN} was correlated with decreased net primary production by the dominant C_4 grass (*Bouteloua gracilis*) and with increased abundance and production by exotic and native C_3 forbs. Reductions in *B. gracilis* may make this system more vulnerable to invasion by exotic species and less tolerant of drought and grazing.

There is general consensus that there is an anthropogenic warming signal in the long-term climate record (*1*). Over land, this is primarily due to average annual minimum temperatures (T_{MIN}) having increased at twice the rate of maximum temperatures (T_{MAX}) (*1*, *2*). At the global scale, these increases in T_{MIN} are related to increases in global cloudiness (*1*, *3*). Experiments with agricultural plants and insect pests suggest important roles for T_{MIN} in influencing plant and insect development (*4*, *5*). However, there has been little research on the consequences of elevated T_{MIN} for natural ecosystems (*6*, *7*). If elevated T_{MIN} leads to longer growing seasons, net primary production and carbon sequestration may increase as a consequence (*8*). However, the opposite may occur if elevated T_{MIN} leads to increased plant and microbial nocturnal respiration rates without a compensatory increase in photosynthesis. Additionally, elevated T_{MIN} could shift competitive interactions among C_3 (cool-season) and C_4 (warm-season) plants.

It is important to identify features of ecosystems that are sensitive to changes in T_{MIN}. To date, most modeling efforts and experimental manipulations investigating ecosystem responses to climate change have assumed that future warming will occur primarily during the day or uniformly over the diurnal cycle. This assumption clearly is not valid on a global level nor at most regional scales (*2*). Furthermore, there is no a priori reason to assume that ecosystems will respond similarly to changes in T_{MIN} and T_{MAX}. To investigate potential ecological consequences of elevated T_{MIN}, we examined a 23-year data set for correlations between temperature [T_{MIN}, T_{MAX}, and mean annual temperature (T_{AVE}) (T_{AVE} = $(T_{MIN} + T_{MAX})/2$)] and both the abundance and aboveground net primary productivity (ANPP) of several key plant species and functional groups found at the Central Plains Experimental Range (*9*) in northeastern Colorado.

We identified seasonal and annual trends in T_{MIN} and T_{MAX} to determine whether asymmetric diurnal temperature increases held true for this site (*10*). The densities of most species were determined by counting all individuals within permanently marked quadrats (*11*). Harvests at time of peak standing crop were used as estimates of ANPP (*12*, *13*). Plants in the shortgrass steppe are commonly water-limited, and variation in precipitation could obscure plant responses to gradually changing temperatures (*9*, *14*). Therefore, we included annual and seasonal precipitation totals, in addition to annual and seasonal minimum and maximum temperatures, as variables for stepwise regression model selection (*15*). We constructed linear models to evaluate significant correlations between these variables and ANPP or plant species density (*16*).

Mean annual temperatures (T_{AVE}) have increased by an average of 0.12°C year^{-1} at this site since 1964 ($P = 0.0001$, $R^2 = 0.52$). During this period, T_{MAX} increased 0.085°C year^{-1} (Fig. 1A), whereas T_{MIN} increased 0.15°C year^{-1} (Fig. 1B). We limited further analyses of temperature to the period beginning in 1970, when standardized monitoring of vegetation density was initiated. Since 1970, T_{AVE} has risen over 1.3°C, largely due to a significant increase in T_{MIN} of 0.12°C year^{-1} ($P = 0.003$; $R^2 = 0.44$). However, there was no significant trend for T_{MAX} ($P = 0.49$). Averages of seasonal minimum temperatures since 1970 also exhibited significant warming, with similar trends in winter (0.17°C year^{-1}, $P = 0.0013$, $R^2 = 0.40$), spring (0.16°C year^{-1}, $P = 0.0007$, $R^2 = 0.43$), and summer T_{MIN} (0.12°C year^{-1}, $P = 0.004$, $R^2 = 0.33$). No significant trends were detected in fall T_{MIN} ($P = 0.64$, $R^2 = 0.01$). Annual precipitation (Fig. 1C) varied from 230 to 480 mm and has also exhibited a significant linear increase since 1970 (6 mm year^{-1}, $P = 0.007$, $R^2 = 0.30$). However, there were no significant correlations between annual or seasonal T_{MIN} and annual or seasonal precipitation ($P > 0.1$).

Since 1983 (*12*), ANPP of *Bouteloua gracilis*, the dominant C_4 grass of the shortgrass steppe, declined over time (–12.2 g m^{-2} year^{-1}; $P = 0.002$; $R^2 = 0.78$), and was negatively correlated with average spring T_{MIN} (Fig. 2A). ANPP of the most abundant C_3 forb, *Sphaeralcea coccinea*, was negatively correlated with winter T_{MIN} (Fig. 2B). In contrast, ANPP of

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Regulation of Murine Cerebral Malaria Pathogenesis by CD1d-Restricted NKT Cells and the Natural Killer Complex

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Summary

NKT cells are specialized cells coexpressing NK and T cell receptors. Upon activation they rapidly produce high levels of interferon-γ (IFN-γ) and interleukin-4 (IL-4) and are therefore postulated to influence T_H1/T_H2 immune responses. The precise role of the CD1/NKT cell pathway in immune response to infection remains unclear. We show here that CD1d-restricted NKT cells from distinct genetic backgrounds differentially influence T_H1/T_H2 polarization, proinflammatory cytokine levels, pathogenesis, and fatality in the *P. berghei ANKA*/rodent model of cerebral malaria. The functional properties of CD1d-restricted NKT cells vary according to expression of loci of the natural killer complex (NKC) located on mouse chromosome 6, which is shown here to be a significant genetic determinant of murine malarial fatalities.

Introduction

About 2.5 million people die of severe *Plasmodium falciparum* malaria every year (World Health Organization, 1992). These fatalities are associated with a spectrum of discrete and overlapping disease syndromes of complex etiologies. Humans affected by malaria may suffer systemic, single-, or multi-organ involvement, including acute respiratory distress, coagulopathy, shock, metabolic acidosis, hypoglycemia, renal failure, pulmonary edema, and cerebral involvement including seizures and coma (White and Ho, 1992). Basic mechanisms controlling these processes are thought to be the site-specific localization of parasites by adherence to vascular endothelial markers such as the adhesin ICAM-1 (Berendt et al., 1989) and both local and systemic inflammatory responses arising from the action of cytokines.

Plasmodium berghei ANKA murine malaria has many features in common with the human disease and is thus an accepted model for certain important aspects of clinical malaria (Miller et al., 2002). It manifests a cytokine-dependent encephalopathy associated with upregulation of adhesins on the cerebral microvascular endothelium and attendant neurological complications (Grau et al., 1987, 1989; Jennings et al., 1997). Particularly in the developmental stages, the murine disease is accepted to reflect the cytokine-dependent inflammatory cascade contributing to cerebral and systemic involvement in humans. In the *P. berghei* ANKA cerebral malaria syndrome, causal roles for tumor necrosis factor-α (TNF-α) and IFN-γ in disease promotion have been established experimentally. Fatalities are abolished by passive transfer of monoclonal antibodies to IFN-γ (Grau et al., 1989) and polyclonal anti-TNF-α (Grau et al., 1987). Mutant mice lacking TNFα/β (Rudin et al., 1997a), lymphotoxin-α (LT-α) (Engwerda et al., 2002), IFN-γ (Rudin et al., 1997b), and TNF-receptor-2 (Lucas et al., 1997) have also been described as being resistant to disease. C57BL6 mice, genetically predisposed toward T_H1-dominated responses (Scott et al., 1989), are susceptible to the murine cerebral malaria syndrome, whereas BALB/c mice, with a genetically determined bias toward T_H2 responses (Scott et al., 1989), are resistant (de Kossodo and Grau, 1993). Thus, susceptibility to experimental cerebral malaria in mice is dependent on the production of proinflammatory and counterregulatory cytokines by T cells and the innate immune system (de Kossodo and Grau, 1993; Grau et al., 1986).

Because cytokine levels become elevated systemically very early during infection with *P. berghei*, it is reasonable to propose that nonconventional lymphoid populations capable of acting with accelerated kinetics may be the source of rapid and high level cytokine output involved in controlling pathology. The identity of such lymphoid sources, however, remains unclear. CD1d-restricted NKT cells are a novel T cell lineage with unusual features (Arase et al., 1992). Upon antigen-specific or polyclonal stimulation through the TCR, CD1d-restricted NKT cells produce large amounts of IL-4 and IFN-γ with rapid kinetics (Yoshimoto and Paul, 1994). They have therefore been postulated to influence T_H1/T_H2 differentiation of the acquired immune system (Porcelli, 1995). However, there is little evidence to date in support of this important proposition. Indeed, the functional significance of the CD1/NKT cell pathway in the immune response to infection is still controversial, as there are few reports to date of clear function for this population in infection models. CD1d-restricted NKT cells have been described to have a role in resistance to *Toxoplasma gondii* (Denkers et al., 1996) and *Leishmania major* (Ishikawa et al., 2000), and to increase susceptibility to *Salmonella* (Naiki et al., 1999) and *Listeria monocytogenes* (Emoto et al., 1995). In this study we sought to investigate the role of CD1-restricted NKT cells in the inflammatory cascade leading to *P. berghei*-mediated cerebral malaria. We found that the CD1d/NKT cell pathway differentially regulates T_H1/T_H2 polarization, malarial pathogenesis, and fatality according to the genetic background of the host. We further show that the natural killer complex (NKC) located on mouse chromosome 6 also regulates NKT cell function, malarial pathogenesis, and fatality. These loci account in part for the differential susceptibility of C57BL6 and BALB/c mice to the *P. berghei* cerebral malaria syndrome.

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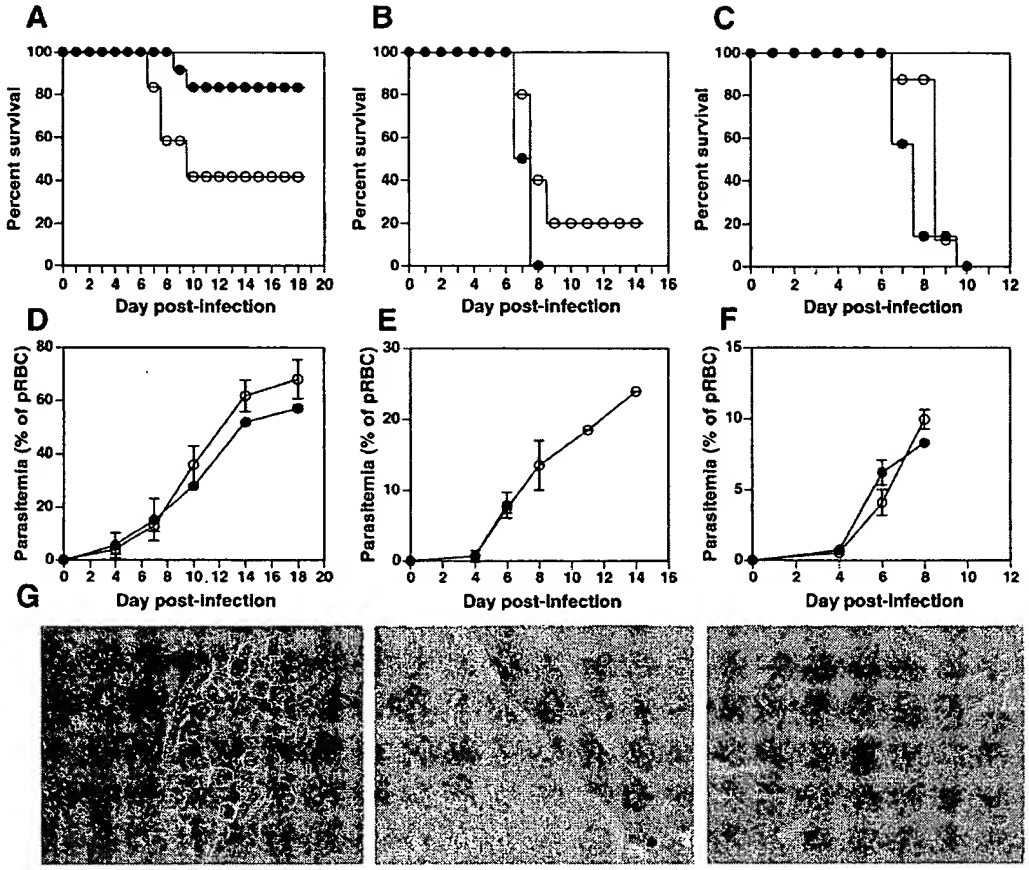


Figure 1. CD1d-Restricted NKT Cells Partially Control Resistance and Susceptibility of Mice to Experimental Cerebral Malaria

WT (closed circles) or mutant mice (open circles) were infected with *P. berghei* ANKA. The percentage survival of BALB/c and CD1$^{-/-}$ BALB/c mice (A), C57BL6 and CD1$^{-/-}$ C57BL6 mice (B), and C57BL6 and Jα281$^{-/-}$ C57BL6 mice (C) was monitored daily. Parasitemia of BALB/c and CD1$^{-/-}$ BALB/c mice (D), C57BL6 and CD1$^{-/-}$ C57BL6 mice (E), and C57BL6 and Jα281$^{-/-}$ C57BL6 mice (F) was assessed from Giemsa-stained blood films. Each point represents the mean parasitemia ±SE of the surviving animals. Each infection is representative of at least four separate experiments. $p < 0.02$, < 0.03, and < 0.05 for (A), (B), and (C), respectively, by Cox-Mantel logrank transformation. (G) Histological examination of brains from C57BL6 WT (left panel), CD1$^{-/-}$ BALB/c (center panel), and BALB/c WT mice (right panel) infected with *P. berghei*. Magnification, ×400.

Results

The CD1d/NKT Cell Pathway Partially Controls Resistance and Susceptibility of Mice to Experimental Cerebral Malaria

To investigate the contribution of CD1d-restricted NKT cells to the pathogenesis of experimental cerebral malaria, mice lacking CD1.1 and CD1.2 (CD1$^{-/-}$) on both the disease-resistant BALB/c and disease-susceptible C57BL6 backgrounds, and wild-type (WT) controls, were challenged with *P. berghei* ANKA, and the course of disease was monitored. Unlike BALB/c WT mice, BALB/c CD1$^{-/-}$ mice developed cerebral malaria ($p < 0.02$), and 60% of the animals died between days 7 to 10 postinfection (p.i.) (Figure 1A). This CD1d deficiency associated with increased susceptibility to disease suggests that in BALB/c mice the CD1/NKT pathway provides protection against cerebral malaria. In marked contrast, CD1d-restricted NKT cells appear to play a modest role in promoting disease in C57BL6 mice, as lack of CD1 partially but significantly ($p < 0.03$) protected against cerebral malaria in this genetic background (Figure 1B). C57BL6 Jα281$^{-/-}$ mice lack the invariant Vα14 T cell receptor (TCR) chain and therefore are specifically deficient in Vα14^{+} NKT cells (Cui et al., 1997). When these mice were challenged with *P. berghei* ANKA, they also showed a significant ($p < 0.05$) delay in the disease onset compared to WT controls (Figure 1C), validating the observations in an independent mutant system and demonstrating a role for the Vα14^{+} NKT population in promoting the disease susceptibility of C57BL6 mice. Parasitemia levels were not affected by deletion of CD1 or Jα281 in either genetic background (Figures 1D–1F). Therefore, control of malarial fatalities by CD1d-restricted NKT cells does not operate through effects on parasite growth rates.

The diagnoses of cerebral malaria were confirmed by histological examination of brains taken at various times p.i. Mice dying of cerebral syndrome displayed typical pathology including high levels of vascular occlusion with both parasitized erythrocytes and macrophages (Figure 1G). This pathology was particularly pronounced

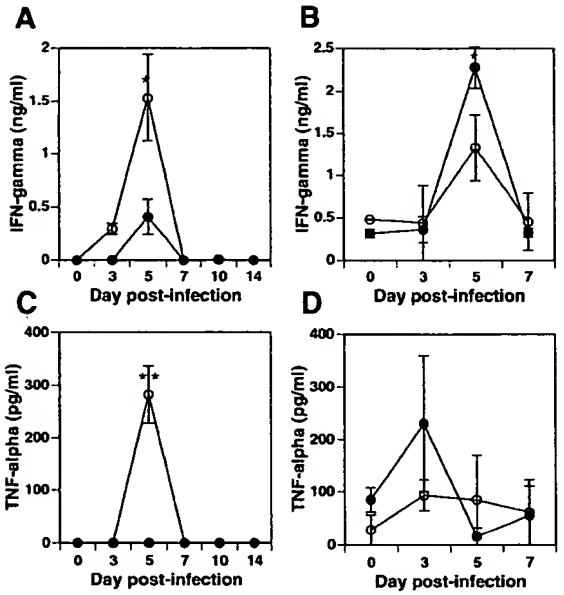


Figure 2. CD1d-Restricted NKT Cells Significantly Control Production of Proinflammatory Cytokines In Vivo in *P. berghei*-Induced Cerebral Malaria

BALB/c (A and C) and C57BL6 (B and D) mice were infected with *P. berghei* ANKA. IFN-γ (A and B) and TNF-α (C and D) levels in sera collected at different time points postinfection from WT (closed circles) or CD1$^{-/-}$ (open circles) mice were measured by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean ±SE. * $p < 0.05$, ** $p < 0.01$ between WT and CD1$^{-/-}$ mice.

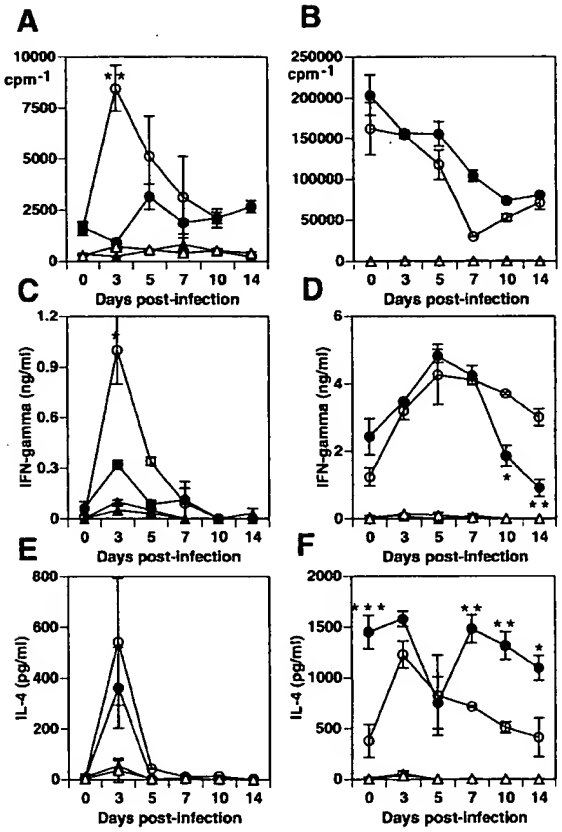


Figure 3. The CD1/NKT Pathway Influences the T_H1/T_H2 Polarization in Response to Malaria

CD4$^+$ T cells from *P. berghei* ANKA-infected BALB/c WT (closed circles) or CD1$^{-/-}$ (open circles) mice were stimulated for 3 days with *P. berghei* ANKA total lysate (A, C, and E) or anti-CD3 (B, D, and F). Cells from WT (closed triangles) and CD1$^{-/-}$ animals (open triangles) were cultured in medium alone as background controls. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (A and B), and IFN-γ (C and D) and IL-4 (E and F) levels in cell culture supernatant by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean of three samples ±SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ between stimulated cells from WT and CD1$^{-/-}$ mice.

in C57BL6 mice and was also present, although with reduced severity, in BALB/c CD1$^{-/-}$ animals. BALB/c WT mice in contrast showed absent or very much reduced vascular occlusion despite similar parasite burdens (Figure 1G). Thus, the presence and severity of histological markers of cerebral disease correlate well with the overall fatality rates in these groups.

The CD1d/NKT Cell Pathway Controls Production of Proinflammatory Cytokines In Vivo in Murine Cerebral Malaria

TNF-α and IFN-γ are two proinflammatory cytokines associated with the development of murine cerebral malaria (Grau et al., 1987, 1989). To study whether CD1d-restricted NKT cells regulate the serum levels of these two cytokines in experimental cerebral malaria, sera were collected from BALB/c and C57BL6 WT or CD1$^{-/-}$ mice at different time points p.i., and the cytokine levels were assessed by capture ELISA. Disease-resistant BALB/c WT mice had negligible levels of serum TNF-α and IFN-γ throughout the course of infection (Figures 2A and 2C). In contrast, TNF-α and IFN-γ levels were significantly higher in more susceptible BALB/c CD1$^{-/-}$ mice at day 5 p.i. (Figures 2A and 2C). IFN-γ was lower in the more resistant CD1$^{-/-}$ C57BL6 as compared with fully susceptible WT C57BL6 mice (Figure 2B). Although showing a trend to reduced TNF-α levels in CD1$^{-/-}$ C57BL6 mice, these results were not significantly different (Figure 2D). Thus, CD1d-restricted NKT cells appear to regulate systemic levels of two proinflammatory cytokines associated with murine malarial pathogenesis, with opposing activities depending on the genetic background of the host.

The CD1d/NKT Cell Pathway Controls the T_H1/T_H2 Balance in Response to Malarial Infection

Based on rapid and high output of IL-4 and IFN-γ, the CD1/NKT pathway has been proposed to influence the regulation of T_H1/T_H2 immune responses (Porcelli, 1995). To investigate whether the differential susceptibility to cerebral malaria in BALB/c and C57BL6 mice reflected the ability of CD1d-restricted NKT cells to influence the T_H1/T_H2 balance in response to infection, we examined cytokine production by CD4$^+$ cells isolated from infected WT and CD1$^{-/-}$ mice. At early stages of the infection, CD4$^+$ cells from mice with increased susceptibility to disease, such as CD1$^{-/-}$ BALB/c, proliferated strongly in response to parasite antigen (Figure 3A) whereas

$CD4^+$ cells from WT mice did not proliferate above background controls. $CD4^+$ cells from $CD1^{-/-}$ mice secreted significantly higher levels of IFN-γ at day 3 p.i. (Figure 3C) compared with disease-resistant BALB/c WT controls. No significant differences between BALB/c WT or $CD1^{-/-}$ mice were detected in the parasite-specific IL-4 production (Figure 3E). After day 7 postinfection, the parasite-induced cell death among antigen-specific lymphocytes reported previously (Hirunpetcharat and Good, 1998) results in a depleted and apparently anergic T cell response, and antigen-specific cytokine and proliferative responses become difficult to elicit in vitro.

To investigate the impact of CD1d-restricted NKT cells on global T cell responses, $CD4^+$ cells from BALB/c WT and $CD1^{-/-}$ mice were stimulated with anti-CD3 antibody. Proliferative responses decreased as the infection developed (Figure 3B), indicating progressive T cell anergy associated with malaria infection. $CD4^+$ cells from both WT and $CD1^{-/-}$ BALB/c mice produced high levels of IFN-γ in response to anti-CD3 antibody during the initial stages of the infection. From day 7 onward, IFN-γ levels decreased in BALB/c WT but remained high in $CD1^{-/-}$ mice (Figure 3D). In contrast, IL-4 production was initially downregulated but markedly increased from day 7 postinfection, demonstrating a switch from T_H1 to T_H2 immune response in BALB/c WT mice. This initial downregulation during early time points followed by recovery of IL-4 production was a reproducible feature of infection in several replicate experiments. Interestingly, in the more susceptible BALB/c $CD1^{-/-}$ mice, IFN-γ production was not downregulated, and no switch to IL-4 production was observed (Figures 3D and 3F), indicating that BALB/c CD1d-restricted NKT cells provide help for the development of T_H2 immune responses.

In contrast to BALB/c mice, CD1d-restricted NKT cells promote both proliferative responses and IFN-γ production in WT C57BL6 mice, as partially protected C57BL6 $CD1^{-/-}$ mice showed reduced antigen-specific (Figure 4A) as well as anti-CD3 mediated (Figure 4B) proliferative responses and reduced IFN-γ levels at early time-points p.i. compared to WT animals (Figures 4C and 4D). No significant differences in IL-4 output were found between $CD4^+$ cells from C57BL6 WT or $CD1^{-/-}$ mice (Figures 4E and 4F).

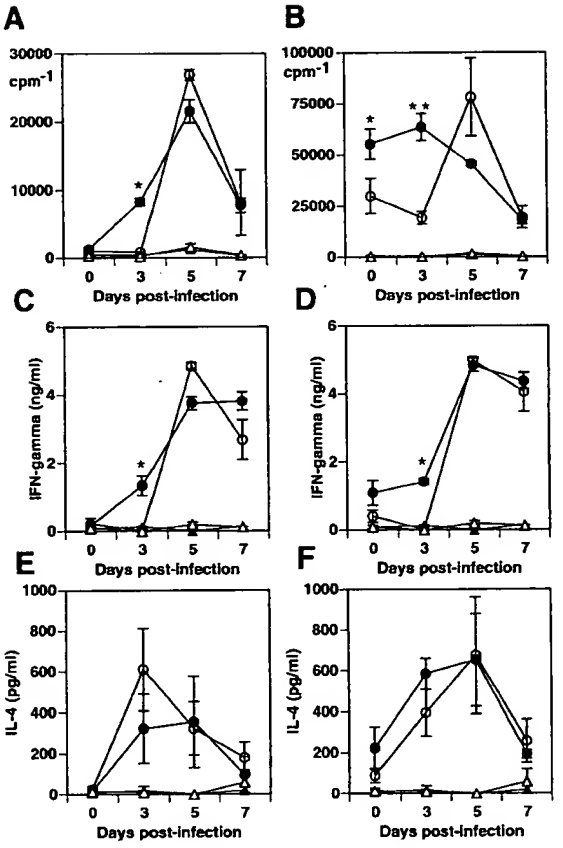
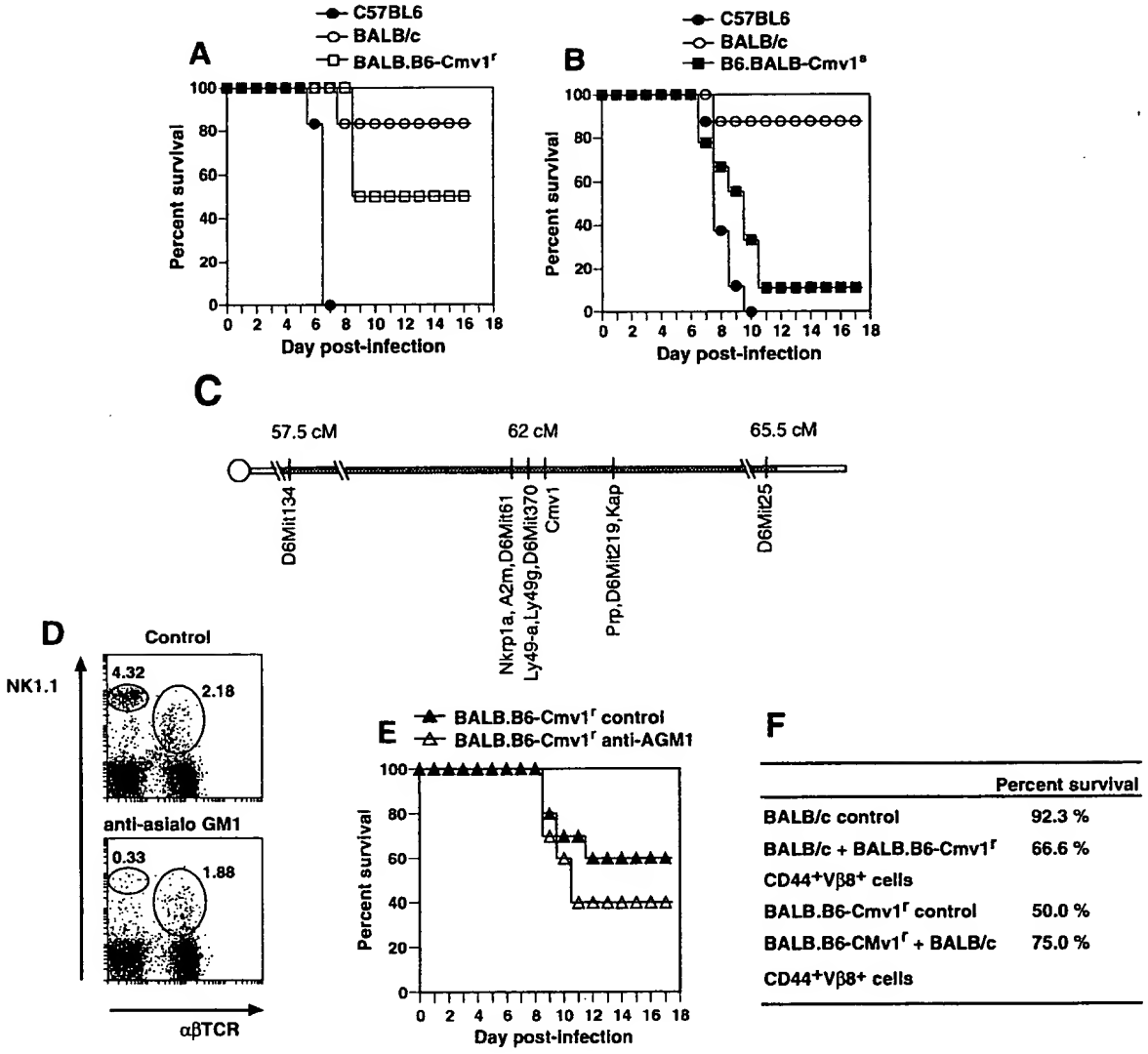


Figure 4. The CD1/NKT Pathway Influences the T_H1/T_H2 Polarization in Response to Malaria

$CD4^+$ T cells from *P. berghei* ANKA-infected C57BL6 WT (closed circles) or $CD1^{-/-}$ (open circles) mice were stimulated for 3 days with *P. berghei* ANKA total lysate (A, C, and E) or anti-CD3 (B, D, and F). Cells from WT (closed triangles) and $CD1^{-/-}$ animals (open triangles) were cultured in medium alone as background controls. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (A and B), and IFN-γ (C and D), and IL-4 (E and F) levels in cell culture supernatant by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean of three samples ±SE. * $p < 0.01$, ** $p < 0.005$ between stimulated cells from WT and $CD1^{-/-}$ mice.

Control of Malarial Pathogenesis by the Natural Killer Complex

Figures 1–4 indicate that the regulatory role of CD1-restricted NKT cells differs markedly between BALB/c and C57BL6 mice. Both NK and NKT cells from C57BL6 and BALB/c mice are known to differ in expression of loci encoded by the NKC (Scalzo et al., 1999). Located on mouse chromosome 6, the NKC comprises several genes (e.g., *Cd69* and *Cd94*) and multigene families (*Nkrp1*, *Nkg2*, and *Ly49*) involved in the activation and inhibition of NK cells. All NKC genes described so far encode type II integral membrane proteins with C-lectin domains having inhibitory or activation activity on cytokine production depending on the presence or absence of immunoreceptor tyrosine-based inhibitory motifs in their intracellular domains. Therefore, NKC products are candidate regulators of malarial pathogenesis. To study whether the NKC genotype can influence the susceptibility to cerebral malaria we made use of NKC homozygous congenic BALB.B6-Cmv1^r mice, in which the region of chromosome 6 containing the NKC from C57BL6 has been introduced onto the BALB/c background (Figure 5C). We also utilized the reverse congenic B6.BALB-Cmv1^s mice, in which C57BL6 background animals bear a 1.5 cM region expressing the NKC of BALB/c mice (Scalzo et al., 1995, 1999). Unlike BALB/c WT mice, BALB.B6-Cmv1^r congenic animals which express the C57BL6 NKC developed cerebral malaria, and 50% of the animals died between days 7 and 9 p.i. In contrast, B6.BALB-Cmv1^s mice were partially protected against disease compared to C57BL6 WT mice (Figures 5A and 5B). Parasitemia levels were not significantly different between WT and congenic mice (data not shown). In addition, histological examination of brains taken from BALB.B6-Cmv1^r animals revealed a typical histological profile consisting of cerebral vasculature occluded with



	Percent survival
BALB/c control	92.3 %
BALB/c + BALB.B6-Cmv1^r CD44$^+$Vβ8$^+$ cells	66.6 %
BALB.B6-Cmv1^r control	50.0 %
BALB.B6-CMv1^r + BALB/c CD44$^+$Vβ8$^+$ cells	75.0 %

Figure 5. Control of Malarial Pathogenesis by the NKC

(A and B) Groups of 10–12 BALB/c, C57BL6, BALB.B6-Cmv1^r, and B6.BALB-Cmv1^s mice were infected with *P. berghei* ANKA. The percentage survival was monitored daily.

(C) Map of the distal region of mouse chromosome 6 in the BALB.B6-Cmv1^r mouse strain. The region inherited by the congenic mice from the C57BL6 background is indicated in gray.

(D) BALB.B6-Cmv1^r mice were depleted of NK cells by i.v injection with anti-asialo GM1 antibody. NK cell depletion was confirmed by FACS.

(E) Groups of ten anti-asialo GM1-treated and control BALB.B6-Cmv1^r mice were infected with *P. berghei* ANKA and percentage survival was monitored daily.

(F) BALB/c mice were injected i.v. with 6.5×10^5 CD44$^+$Vβ8$^+$ cells (sorted as shown in Figure 7A) from malaria-infected BALB.B6-Cmv1^r animals, and BALB.B6-Cmv1^r mice received CD44$^+$Vβ8$^+$ cells from malaria-infected BALB/c wild-types. Adoptively transferred mice and controls were then challenged with *P. berghei* ANKA. The percentage survival was monitored daily.

both parasitized erythrocytes and macrophages as found previously in BALB/c CD1$^{-/-}$ mice (data not shown). Thus, the NKC is a significant determinant of murine cerebral malaria and, in common with the CD1d/NKT pathway, imparts either protective or disease-enhancing properties depending on genotype.

NKC loci are expressed on NK cells as well as NKT cells. To determine which population was responsible for mediating NKC-dependent regulation of malarial pathogenesis, BALB.B6-Cmv1^r mice were treated with anti-asialoGM1 antibody, known specifically to deplete NK cells without affecting NKT cells (Smyth et al., 2001). Depletion of NK cells in BALB.B6-Cmv1^r mice, confirmed by FACS analysis (Figure 5D), did not reverse their increased susceptibility to cerebral malaria ($p > 0.38$) (Figure 5E). To further investigate whether the expression of NKC markers influences the regulation of malarial pathogenesis by NKT cells, CD44$^+$Vβ8$^+$ NKT cells from BALB.B6-Cmv1^r or BALB/c infected donors (purified by FACS sorting) were adoptively transferred into naive recipient mice. CD44$^+$Vβ8$^+$ cells from BALB.B6-Cmv1^r mice transferred into BALB/c recipients

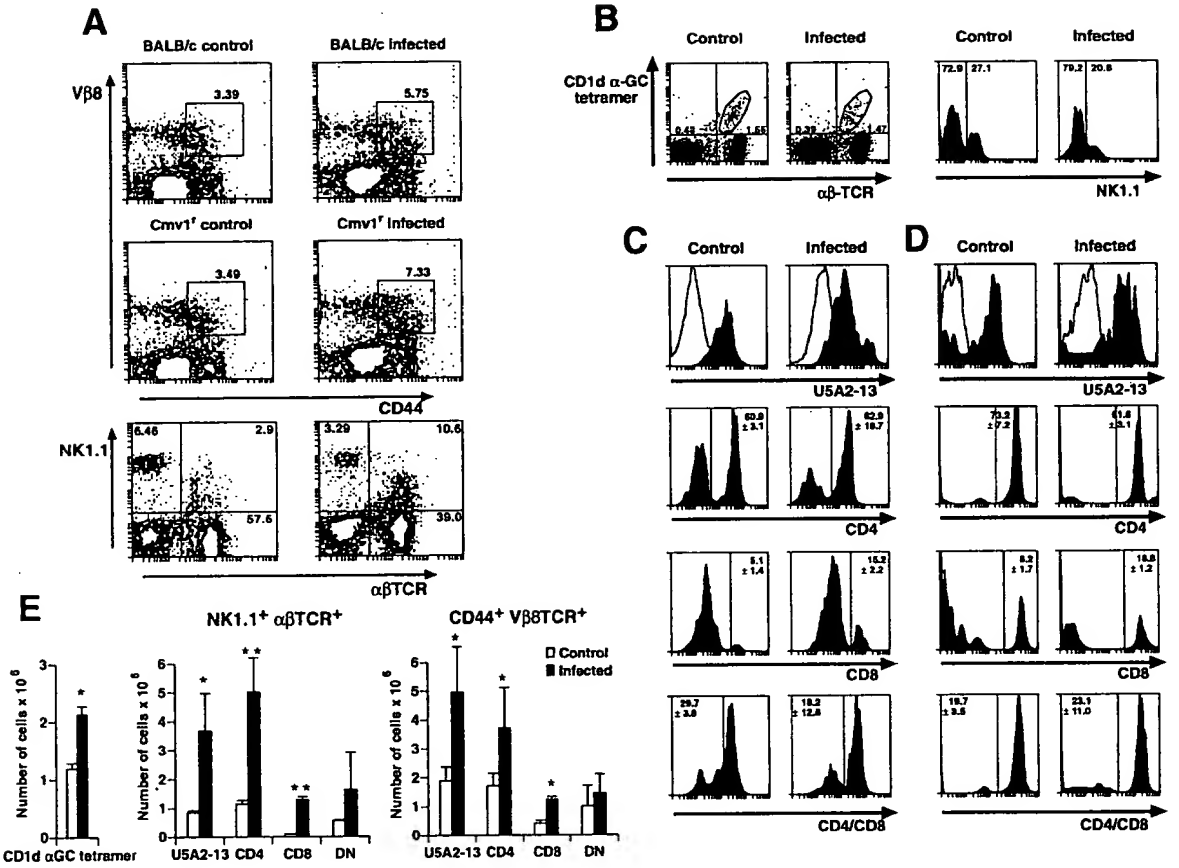


Figure 6. Expansion of Splenic NKT Cells in Response to *P. berghei* ANKA Infection

(A) Spleen cells from naive or infected BALB/c and BALB.B6-Cmv1r mice were stained with anti-CD44 and anti-Vβ8TCR antibodies (top and center panels). BALB.B6-Cmv1r splenocytes were also stained with anti-NK1.1 and anti-TCRαβ antibodies (bottom panel).

(B) BALB.B6-Cmv1r splenocytes were stained with CD1d α-GalCer tetramer and anti-TCRαβ antibodies. The expression of NK1.1+ cells was analyzed in the double-positive population indicated.

(C and D) BALB.B6-Cmv1r splenocytes were stained with anti-NK1.1 and anti-TCRαβ antibodies (C) or with anti-CD44 and anti-Vβ8TCR antibodies (D). The expression of U52A-13, CD4, CD8, and CD4/CD8 positive cells was analyzed in the double-positive populations (shaded histograms). Empty histograms represent staining in gated NK1.1− or Vβ8− T cells.

(E) The absolute number α-GalCer tetramer+-TCRαβ+ and NK1.1+TCRαβ+ or CD44+Vβ8+ subsets in the spleens of malaria infected or naive BALB.B6-Cmv1r mice. Each bar represents the mean of three to five mice ±SE. * $p < 0.05$, ** $p < 0.005$ between cells from infected mice and controls.

increased their susceptibility to *P. berghei*-mediated cerebral malaria (Figure 5F). In contrast, CD44+Vβ8+ cells from malaria-infected BALB/c mice were able to transfer partial resistance to BALB.B6-Cmv1r animals (Figure 5F). As predicted, the reversal of phenotypes was partial, due possibly to the competing contribution of resident NKT cells in recipients. Thus, NKC expression on NKT cells may be responsible for the increased susceptibility to disease of BALB.B6-Cmv1r mice.

A Heterogeneous NK1.1+ αβTCR+ Population Expands in Response to *P. berghei* Infection

We sought to determine whether NKT cells were expanded in response to *P. berghei* infection. Figure 6A shows a 70% and 100% increase in the percentage of Vβ8+CD44+ cells in the spleens of BALB/c and BALB.B6-Cmv1r mice, respectively, at day 3 p.i. The percentage of NK1.1+αβTCR+ cells increased greatly in the spleens of infected BALB.B6-Cmv1r mice (to 10.6%) compared to noninfected (2.9%) control animals (Figure 6A) reaching a peak at day 5 p.i. To undertake further phenotyping, splenocytes from BALB.B6-Cmv1r naive and infected (day 5 p.i.) mice were stained with various antibodies and with CD1d α-Galactosylceramide (α-GalCer) tetramers. The absolute number of CD1d α-GalCer tetramer+ αβTCR+ NKT cells doubled in response to malarial infection (Figure 6E). The relative proportion of +αβTCR+ CD1d α-GalCer tetramer+ cells, however, did not change in spleens of infected mice compared to controls (Figure 6B). Interestingly, although splenic CD1d α-GalCer tetramer+ cells were expanded, the proportion of these cells that were NK1.1+ was slightly reduced in infected animals (Figure 6B). We also gated NK1.1+αβTCR+ (Figure 6C) or Vβ8+CD44+ cells (Figure 6D) from naive and infected BALB.B6-Cmv1r mice (day 5 p.i) and examined expression of the pan NK-

NKT cell marker U5A2-13, CD4, and CD8. The relative proportions of U5A2-13$^+$ (60%–80%), CD4$^+$ (60%–70%), and DN cells (20%–30%) were similar in spleens from naive and infected mice (Figures 6C and 6D). However, the absolute number of these NKT cell subsets substantially increased in response to infection (Figure 6E). Both the percentage and absolute number of CD8$^+$ cells among the NK1.1$^+\alpha\beta$TCR$^+$ and Vβ8$^+$CD44$^+$ cells were significantly higher in infected compared to noninfected control mice (Figures 6C–6E), although this expansion was not alone sufficient to account for the absolute increase in these populations. CD8 T cells, carrying the TCR Vβ8$^+$, have been previously described to expand in response to malaria infection (Boubou et al., 1999). Taken together, these results indicate that a heterogeneous NK1.1$^+\alpha\beta$TCR$^+$ population including conventional and nonconventional NKT cells as well as CD8 T lymphocytes expands in response to malaria infection.

The NKC Phenotype Influences the Capacity of NKT Cells to Secrete Cytokines

To determine whether NKC phenotype can directly influence the capacity of NKT cells to secrete cytokines, Vβ8$^+$CD44$^+$ NKT cells were isolated by FACS sorting from splenocytes of BALB/c, C57BL6, and BALB.B6-Cmv1^r mice (Figure 7A). The cells were then stimulated for 4 days with plate-bound anti-CD3 or anti-NK1.1 antibodies. When sorted Vβ8$^+$CD44$^+$ cells were cultured with anti-CD3, they secreted high levels of both IL-4 and IFN-γ (Figures 7B and 7C). Stimulation with anti-NK1.1 antibody induced only IFN-γ production but no IL-4 in NKT cells from C57BL6 and BALB.B6-Cmv1^r mice (Figures 7B and 7C). Thus, stimulation through the TCR results in comparable levels of both IFN-γ and IL-4 output in BALB/c and C57BL6 mice. However, activation of NKC receptors such as NK1.1 expressed in the C57BL6 background preferentially induces IFN-γ production.

To investigate whether the NKC phenotype also influences the ability of NKT cells to secrete cytokines in response to malaria infection, sorted Vβ8$^+$CD44$^+$ (Figure 7A) cells from *P. berghei*-infected BALB/c and BALB.B6-Cmv1^r mice were stimulated with α-GalCer, and proliferative responses and IL-4 and IFN-γ production were analyzed. Vβ8$^+$CD44$^+$ cells from both BALB/c and BALB.B6-Cmv1^r mice proliferated in response to α-GalCer, confirming the presence of Vα14$^+$ NKT cells within that population (Figure 7D). In both mouse strains, cell proliferation decreases as the infection develops, suggesting that CD1d-restricted NKT cells do not evade the progressive T cell anergy associated with malaria infection. Like cell proliferation, cytokine output gradually decreases in response to infection. α-GalCer-stimulated NKT cells from both infected BALB/c and BALB.B6-Cmv1^r mice produced similar levels of IL-4 (Figure 7E). By days 7 and 10 p.i., however, BALB/c NKT cells cease IFN-γ production almost entirely. In contrast, NKT cells from BALB.B6-Cmv1^r mice continued to secrete around 15 times more IFN-γ than NKT cell from BALB/c mice (Figure 7F). The ex vivo cytokine production by NKT cells from infected mice was also analyzed. To that end, splenocytes from infected mice were stained with anti-CD44 and anti-Vβ8, and intracellular IL-4 and IFN-γ were analyzed by FACS on gated CD44$^+$Vβ8$^+$. CD44$^+$Vβ8$^+$cells from BALB.B6-Cmv1^r mice displayed nearly three times higher IFN-γ levels than cells from BALB/c mice (Figure 7G). IL-4 responses were in general low, and no differences were found between BALB/c and BALB.B6-Cmv1^r mice. Similar results were obtained when intracellular cytokines were analyzed on gated U5A2-13$^+$TCR$^+$ cells (data not shown). Thus, in malaria infection, expression of C57BL6 NKC markers predisposes NKT cells to increased IFN-γ production. These data are highly concordant with the proposition that differential expression of NKC loci may account in part for the differential immunological behavior of the CD1/NKT cell pathway in C57BL6 and BALB/c mice.

Discussion

This study shows that CD1d-restricted NKT cells influence the T_H1/T_H2 balance, pathogenesis, and fatality in murine cerebral malaria, and cells from distinct genetic backgrounds appear to have opposing properties in this regard. The CD1/NKT cell pathway appears to favor the development of T_H2 polarization and resistance to cerebral malaria in BALB/c mice, and induces early IFN-γ production and promotes pathology in susceptible C57BL6 animals. CD1d-restricted NKT cells clearly influence the overall production of IFN-γ and TNF-α in vivo during malaria infection. The differential expression of molecules encoded by the NKC may account in part for the opposing roles of NKT cells in C57BL6 and BALB/c mice in response to *P. berghei* infection. Our data, moreover, indicate that the NKC is a significant genetic determinant of murine cerebral malaria, imparting partial protection or susceptibility depending on genotype.

Throughout this study, the most pronounced effects in relation to both CD1d-restricted NKT cells and the NKC in protection against cerebral malaria were observed on the BALB/c background, compared with a smaller but significant and reproducible effect in promoting disease susceptibility and T_H1 responses in C57BL6 mice. This differential penetrance presumably reflects a relatively greater contribution by other susceptibility loci in the latter case, i.e., it seems likely that additional pathways or loci promote disease susceptibility in C57BL6 mice.

Previous studies in rodent malaria infections show that an initial T_H1 response switches after the first week to a T_H2 response (Langhorne et al., 1989; Taylor-Robinson et al., 1993). The sequential onset of T_H1-mediated cellular mechanisms and T_H2-mediated antibody responses is proposed to be necessary for the immunological control of *Plasmodium* blood stage infections. Our data indicate that a similar process occurs in *P. berghei*-infected disease-resistant BALB/c mice and that this is regulated by CD1d-restricted NKT cells. In this case, the switch from T_H1 to T_H2 immune responses may downregulate the strong proinflammatory response involved in the cerebral syndrome, explaining a protective role against disease for CD1d-restricted NKT cells in BALB/c mice. These findings from an infectious disease are consistent with data from a T_H1-driven autoimmune condition, where adoptive transfer or overexpres-

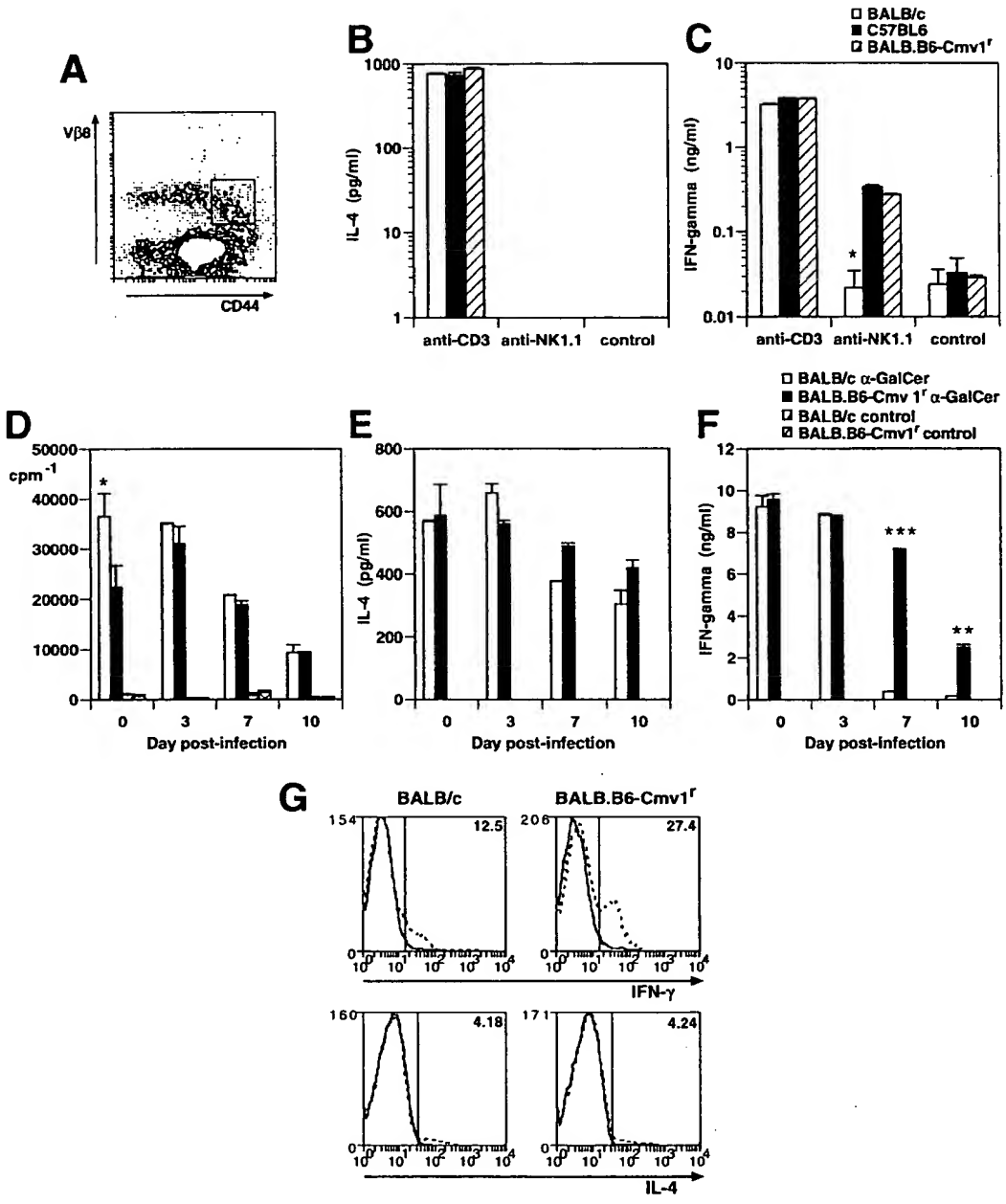


Figure 7. The NKC Phenotype Influences the Capacity of NKT Cells to Secrete Cytokines

(A) The Vβ8$^+$CD44$^+$ population of splenic NKT cells was purified by FACS sorting from splenocytes of BALB/c, C57BL6, and BALB.B6-Cmv1^r mice. The purified NKT cells were stimulated for 4 days with plate-bound anti-CD3, anti-NK1.1, or medium alone.

(B and C) IFN-γ (B) and IL-4 (C) levels were measured in cell culture supernatants by ELISA. Each bar represents the mean of three samples ±SE. * $p < 0.05$ between BALB/c and C57BL6 or BALB.B6-Cmv1^r mice.

(D–F) Vβ8$^+$CD44$^+$ NKT cells from *P. berghei*-infected BALB/c and BALB.B6-Cmv1^r mice were stimulated with α-GalCer for 4 days. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (D), and IL-4 (E) and IFN-γ (F) levels in cell culture supernatant by capture ELISA. Each bar represents the mean of three samples ±SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ between cells from α-GalCer-stimulated BALB/c and BALB.B6-Cmv1^r mice.

(G) Ex vivo cytokine production by NKT cells. Splenocytes from BALB/c and BALB.B6-Cmv1^r malaria infected-mice were stained with anti-CD44 and anti-Vβ8TCR antibodies, and intracellular IL-4 or IFN-γ was analyzed by FACS. Representative histograms from cells gated on CD44$^+$Vβ8$^+$ are shown. The dotted lines represent cytokine staining from infected mice (day 7), and the full lines show noninfected controls.

sion of NKT cells in nonobese diabetic (NOD) mice protects against diabetes (Hammond et al., 1998; Lehuen et al., 1998). Furthermore, defects in NKT cell number and function occur in murine and human type I diabetes (Godfrey et al., 1997; Wilson et al., 1998), and CD1$^{-/-}$ NOD mice are more susceptible to the disease (Shi et al., 2001).

Genetically determined resistance to murine cyto-

megalovirus (MCMV) is controlled by a gene designated *Cmv1* that regulates MCMV replication in the spleens of mice (Scalzo et al., 1995). C57BL6 mice express the *Cmv1*^{r} allele and are resistant to this condition whereas BALB/c mice express the *Cmv1*^{s} allele and show high viral titers. Generation of congenic mouse strains has allowed the mapping of these alleles to the NKC located on the distal region of mouse chromosome 6. Recent studies show that *Cmv1*^{r} maps to the NK activation receptor Ly49H (Dokun et al., 2001). Molecules encoded by the NKC include different receptors involved in the activation or inhibition of NK cells. They are all type II integral proteins with C-lectin domains. The NK1.1 alloantigen is an activation receptor. Its function is still unknown but it has been shown that stimulation with anti-NK1.1 antibody induces NK cell-mediated cytotoxicity and cytokine production (Karlhofer and Yokoyama, 1991). Furthermore, this report and others (Arase et al., 1996) show that crosslinking of NK1.1 preferentially induces IFN-γ and no IL-4 production by CD1d-restricted NKT cells, raising the possibility that carbohydrate recognition by these cells is involved in upregulation of proinflammatory responses by a TCR-independent pathway. Other multigene families encoded within the NKC include Ly49 and NKG2 receptors. Protein products encoded by these genes are responsible for the recognition of MHC class I molecules on potential target cells and subsequent inhibition or activation of cytotoxic activity (Lanier, 1998). Stimulation of activation receptors such as Ly49D (Mason et al., 2000) and Ly49H (Dokun et al., 2001) leads to IFN-γ secretion. Moreover, a recent study demonstrated that a subset of NKT cells expressing high levels of NKG2d selectively produces IFN-γ and TNF-α (Gumperz et al., 2002). Together, this evidence suggests that expression of NKC markers on NKT cells might provide additional pathways for induction of T_H1-like responses.

Most malaria susceptibility/resistance loci described in humans are involved in the control of parasite replication. In murine systems, a congenic study has defined a role for MHC loci in determining resistance to *P. chabaudi* infection (Wunderlich et al., 1988). Quantitative trait locus (QTL) analyses on genome scans from murine intercrosses identify alleles on chromosomes 8 and 9 (*char1* and *char2*) with 30%–40% penetrance in controlling *P. chabaudi* replication (Foote et al., 1997; Fortin et al., 1997). The present study uses congenic mice to establish one or more NKC loci on mouse chromosome 6 exerting 40%–50% penetrance in the control of *P. berghei*-induced pathogenesis and fatality through mechanisms independent of parasite replication. The murine NKC and human syntenic regions constitute significant regulatory components of innate immunity, exhibiting a high level of functional polymorphisms in both species. This evidence therefore allows formulation of the testable hypotheses that syntenic human chromosomal regions corresponding to *char1*, *char2*, and the NKC may be associated with resistance or susceptibility to pathogenesis and fatalities in human malaria.

Much useful evidence to date concerning NKT cell function in vivo is provided by the exogenous administration of α-GalCer (Kawano et al., 1997). However, this glycolipid antigen stimulates only through the TCR. *P. berghei* ANKA infection induces a generalized activation of the immune system and provides a framework allowing the characterization of the NKT cell function under physiological conditions. This model provides evidence that NKT cells can be stimulated by pathways other than the TCR and that the genetic background of the host influences the regulatory impact of NKT cells on the global immune response. Moreover, this study and other infection models including *Leishmania major* (Ishikawa et al., 2000), Hepatitis B virus (Baron et al., 2002), *Crytococcus neoformans* (Kawakami et al., 2001), and *Salmonella* (Naiki et al., 1999) indicated that NKT cell populations become highly expanded in response to infection. In contrast, stimulation with glycolipids like α-GalCer results in strong TCR-driven NKT cell activation (Kawano et al., 1997), followed by massive NKT cell death and homeostatic replacement from the bone marrow (Eberl and MacDonald, 2000). It is therefore possible that stimulation of NKT cells with physiological ligands expressed during viral, bacterial, or parasitic infections results in alternative or additional activation pathways. Studies aimed at identification and characterization of NKT cell natural ligands are necessary to address this proposition.

In addition to α-GalCer, CD1d-restricted NKT cells recognize GPIs of parasite origin when these agents are supplied as exogenous antigens (Schofield et al., 1999) but not when expressed as endogenous ligands within the antigen-presenting cell (Molano et al., 2000). Malarial GPI comprises greater than 95% of plasmodial carbohydrate, is the main posttranslational modification of parasite antigens (Gowda et al., 1997), and is therefore a reasonable candidate antigenic stimulus for NKT cells in malaria infection. NKT cells may be influenced toward a T_H2 polarized cytokine profile by immunization with α-GalCer (Burdin et al., 1999; Singh et al., 1999). These considerations raise the possibility of reducing susceptibility to severe malarial disease in human populations by immunomodulation via a T_H2-biased, CD1-restricted glycolipid vaccine.

Experimental Procedures

Mice and Infections

Eight- to twelve-week-old BALB/c, BALB/c CD1$^{-/-}$ (F_{10} generation [Smiley et al., 1997]), C57BL6, C57BL6 CD1$^{-/-}$ (F_6 generation [Sonoda et al., 1999]), C57BL6 Jα281$^{-/-}$ (F_9 generation [Cui et al., 1997]), BALB.B6-Cmv1^r (F_6 generation [Scalzo et al., 1995]), and B6.BALBCmv1^s (F_6 generation [Scalzo et al., 1999]) mice were used throughout the study. The BALB.B6-Cmv1^r and B6.BALBCmv1^s mice were backcrossed using speedy marker-assisted congenic mapping methods. Groups of 10–15 BALB/c WT, BALB/c CD1$^{-/-}$, BALB.B6-Cmv1^r, and B6.BALBCmv1^s were injected i.p. with 1×10^6, and C57B/6 WT, C57B/6 CD1$^{-/-}$, and Jα281$^{-/-}$ mice received 1×10^4, *P. berghei* ANKA-infected erythrocytes. In some experiments, mice were injected i.v. with 20 μg of anti-asialo GM1 antibody (Wako, Osaka, Japan) 6 hr before challenge in order to deplete NK cells. NK cell depletion was confirmed by flow cytometry. For adoptive transfer experiments, mice were injected i.v. with 6.5×10^5 CD44$^+$Vβ8$^+$ cells purified from infected mice (day 7 p.i.) by FACS sorting as described below. Control mice received saline. Four hours later, adoptively transferred mice and controls were challenged with *P. berghei* ANKA. Parasitemia was assessed from Giemsa-stained smears of tail blood prepared every 2–3 days. Mortality was checked daily. Mice were judged as developing cerebral malaria if they displayed neurological signs such as ataxia, loss of reflex, and hemiplegia, and died between days 6 to 12 postinfection with relatively low parasitemia. Statistical differences in mortality rates of groups of *P.*

berghei infected mice during this period of susceptibility to cerebral malaria were assessed by Cox-Mantel logrank analysis. All experiments were performed in compliance with local Animal Ethics Committee requirements.

Histology

For histological analysis of cerebral pathology, brains were taken into 10% neutral-buffered formalin, sectioned (5 μm), and stained with hemotoxylin and eosin. Slides were coded and scored blind for histological evidence of cerebral syndrome.

Lymphoproliferative Assays

Spleen cells from BALB/c and C57BL6, WT and $CD1^{-/-}$ mice (n = 3) were collected at different times postinfection with *P. berghei* ANKA. Splenic $CD4^+$ cells were purified by positive selection with Dynabeads following the manufacturer's instructions (98%–99% purity) (Dynal Biotech, Norway). For proliferation assays, $CD4^+$ cells suspended in complete RPMI-1640 medium, 5% fetal calf serum, were seeded in 96-well plates at a density of 5×10^5 cells/ml. Naive syngeneic spleen cells irradiated to 3000 rads were added as antigen-presenting cells at a density of 2×10^6 cells/ml. Cells were then stimulated in triplicate for 3 days with *P. berghei* ANKA total lysate (50 μg/ml) or anti-CD3 (5 μg/ml, Pharmingen, San Diego, CA). Cells cultured in medium alone were used as background controls. [Methyl-^{3}H]-thymidine (2 μCi/well, 5 Ci/mmol, Amersham, UK) was added 16 hr before harvest, and radioactivity was measured in a betaplate counter.

ELISA for IL-4, IFN-γ, and TNF-α Detection

The following pairs of antibodies were used: 11B11 for capture and BVD6-24G2 for detection of IL-4; R4-6A2 for capture and XMG1-2 for detection of IFN-γ, and G281-2626 for capture and MP6-XT3 for detection of TNF-α (all antibodies from Pharmingen, San Diego, CA). Antibodies used for detection were biotinylated. Ninety-six-well plates were coated with capture antibody by overnight incubation at 4°C in phosphate buffer (pH 9) for IL-4 and IFN-γ, and phosphate buffer (pH 6) for TNF-α. Plates were then blocked with 1% BSA for 1 hr at 37°C. Splenocyte culture supernatants or sera from infected mice were tested in duplicate by overnight incubation at 4°C under mild agitation. The plates were then incubated for 3 hr at 20°C with the respective biotinylated antibody followed by a 2 hr incubation at 20°C with streptavidin-peroxidase conjugate (Pierce, Rockford, IL). Bound complexes were detected by reaction with tetramethylbenzidine (KBL, Gaithersburg, MD) and H_2O_2. Absorbance was read at 450 nm. The cytokine concentration in samples was calculated as pg/ml using recombinant murine cytokines (Pharmingen, San Diego, CA) for the preparation of standard curves.

Flow Cytometry

Spleen cells from BALB/c and BALB.B6-Cmv1^{-r} mice were incubated with anti-CD16 antibody (Fc-block), washed, and stained with NK1.1-PE or FITC-conjugated together with anti-αβTCR-FITC or CyChrome-conjugated antibodies for 1 hr on ice. NKT cells were also characterized by staining with anti-CD44-PE or CyChrome-conjugated and anti-Vβ8 TCR-FITC-conjugated antibodies. In some experiments cells were stained with additional antibodies, i.e., FITC- or PE-conjugated anti-CD4, FITC-, or PE-conjugated anti-CD8, PE-conjugated U5A2-13 (all antibodies from Pharmingen, San Diego, CA), and CD1d α-GalCer tetramer (kind gift of Dr. M. Kronenberg). The cells were then washed two times with PBS containing 1% FCS and suspended in 200 μl of PBS. For intracellular cytokine staining, spleen cells from infected mice were incubated with Fc-block, washed, and stained with surface markers as described above. The cells were fixed and permeabilized with Citofix/Citoperm (Pharmingen, San Diego, CA), and incubated with anti-IFN-γ-FITC or anti-IL-4-PE conjugated antibodies. Appropriate isotype-matched controls were included. The stained cells were then analyzed by FACScan. Dead cells were gated out by forward and side scatter.

Purification and Stimulation of Splenic NKT Cells

Spleen cells from naive or *P. berghei*-infected BALB/c, C57BL6, and BALB.B6-Cmv1^{-r} mice were incubated with anti-CD16 antibody washed and stained with anti-CD44-PE conjugated antibody and anti-Vβ8 TCR-FITC-conjugated antibody (all antibodies from Pharmingen, San Diego, CA) for 1 hr on ice. After washing two times with PBS, double-positive cells were isolated by flow cytometry. The purified cells were seeded in triplicates in 96-well plates at a density of 3×10^4 cells and stimulated with plate-bound anti-CD3 (10 μg/ml), plate-bound anti-NK1.1 (100 μg/ml), or with α-GalCer, (50 ng/ml) for 4 days. α-GalCer was kindly provided by the Pharmaceutical Research Laboratories, Kirin Brewery (Gumna, Japan). In α-GalCer-stimulated cultures, naive syngeneic spleen cells depleted of T cells with Dynabeads (Dynal Biotech, Norway) and irradiated to 3000 rads were added as antigen-presenting cells at a density of 2×10^6 cells/ml. Cells cultured in medium alone were used as background controls. [Methyl-^{3}H]-thymidine (2 μCi/well, 5 Ci/mmol, Amersham, UK) was added 16 hr before harvest, and radioactivity was measured in a betaplate counter. The cell culture supernatants were collected to measure the production of IL-4 and IFN-γ by capture ELISA.

Statistic Analysis

A paired-sample Student's t test was used for data evaluation. Differences in mortality rates of *P. berghei*-infected mice during the period of susceptibility were assessed by Cox-Mantel logrank analysis.

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References

Arase, H., Arase, N., Ogasawara, K., Good, R.A., and Onoe, K. (1992). An $NK1.1^+$ $CD4^+8^-$ single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor Vβ family. Proc. Natl. Acad. Sci. USA *89*, 6505–6510.

Arase, H., Arase, N., and Saito, T. (1996). Interferon γ production by natural killer (NK) cells and $NK1.1^+$ T cells upon NKR-P1 cross-linking. J. Exp. Med. *183*, 2391–2396.

Baron, J.L., Gardiner, L., Nishimura, S., Shinkai, K., Locksley, R., and Ganem, D. (2002). Activation of a nonclassical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. Immunity *16*, 583–594.

Berendt, A.R., Simmons, D.L., Tansey, J., Newbold, C.I., and Marsh, K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion molecule for *Plasmodium falciparum*. Nature *341*, 57–59.

Boubou, M.I., Collette, A., Voegtle, D., Mazier, D., Cazenave, P.A., and Pied, S. (1999). T cell response in malaria pathogenesis: selective increase in T cells carrying the TCR V(beta)8 during experimental cerebral malaria. Int. Immunol. *11*, 1553–1562.

Burdin, N., Brossay, L., and Kronenberg, M. (1999). Immunization with α-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur. J. Immunol. *29*, 2014–2025.

Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y., Koseki, H., Kanno, M., and Taniguchi, M. (1997). Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. Science *278*, 1623–1626.

de Kossodo, S., and Grau, G.E. (1993). Profiles of cytokine production in relation with susceptibility to cerebral malaria. J. Immunol. *151*, 4811–4820.

Denkers, E.Y., Scharton-Kersten, T., Barbieri, S., Caspar, P., and Sher, A. (1996). A role for CD4+ NK1.1+ T lymphocytes as major histocompatibility complex class II independent helper cells in the generation of CD8+ effector function against intracellular infection. J. Exp. Med. *184*, 131–139.

Dokun, A.O., Kim, S., Smith, H.R., Kang, H.S., Chu, D.T., and Yokoyama, W.M. (2001). Specific and nonspecific NK cell activation during virus infection. Nat. Immunol. *2*, 951–956.

Eberl, G., and MacDonald, H.R. (2000). Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. Eur. J. Immunol. *30*, 985–992.

Emoto, M., Emoto, Y., and Kaufmann, S.H. (1995). Interleukin-4-producing CD4+ NK1.1+ TCR alpha/beta intermediate liver lymphocytes are down-regulated by Listeria monocytogenes. Eur. J. Immunol. *25*, 3321–3325.

Engwerda, C.R., Mynott, T.L., Sawhney, S., De Souza, J.B., Bickle, Q.D., and Kaye, P.M. (2002). Locally upregulated lymphotoxin-alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J. Exp. Med. *195*, 1371–1377.

Foote, S.J., Burt, R.A., Baldwin, T.M., Presente, A., Roberts, A.W., Laural, Y.L., Lew, A.M., and Marshall, V.M. (1997). Mouse loci for malaria-induced mortality and the control of parasitaemia. Nat. Genet. *17*, 380–381.

Fortin, A., Belouchi, A., Tam, M.F., Cardon, L., Skamene, E., Stevenson, M.M., and Gros, P. (1997). Genetic control of blood parasitaemia in mouse malaria maps to chromosome 8. Nat. Genet. *17*, 382–383.

Godfrey, D.I., Kinder, S.J., Silvera, P., and Baxter, A.G. (1997). Flow cytometric study of T cell development in NOD mice reveals a deficiency in α-β-TCR$^+$CD4$^-$CD8$^-$ thymocytes. J. Autoimmun. *10*, 279–285.

Gowda, D.C., Gupta, P., and Davidson, E.A. (1997). Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage *Plasmodium falciparum*. J. Biol. Chem. *272*, 6428–6439.

Grau, G.E., Piguet, P.F., Engers, H.D., Louis, J.A., Vassalli, P., and Lambert (1986). L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J. Immunol. *137*, 2348–2354.

Grau, G.E., Fajardo, L.F., Piguet, P.F., Allet, B., Lambert, P.H., and Vassalli, P. (1987). Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science *237*, 1210–1212.

Grau, G.E., Heremans, H., Piguet, P.F., Pointaire, P., Lambert, P.H., Billiau, A., and Vassalli, P. (1989). Monoclonal antibody against interferon γ can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. Proc. Natl. Acad. Sci. USA *86*, 5572–5574.

Gumperz, J.E., Miyake, S., Yamamura, T., and Brenner, M.B. (2002). Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J. Exp. Med. *195*, 625–636.

Hammond, K.J.L., Poulton, L.D., Palmisano, L.J., Silveira, P.A., Godfrey, D.I., and Baxter, A.G. (1998). α/β-T cell receptor (TCR)$^+$CD4$^-$CD8$^-$ (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. J. Exp. Med. *187*, 1047–1056.

Hirunpetcharat, C., and Good, M.F. (1998). Deletion of *Plasmodium berghei*-specific CD4+ T cells adoptively transferred into recipient mice after challenge with homologous parasite. Proc. Natl. Acad. Sci. USA *95*, 1715–1720.

Ishikawa, H., Hisaeda, H., Taniguchi, M., Nakayama, T., Sakai, T., Maekawa, Y., Nakano, Y., Zhang, M., Zhang, T., Nishitani, M., et al. (2000). CD4(+) v(alpha)14 NKT cells play a crucial role in an early stage of protective immunity against infection with Leishmania major. Int. Immunol. *12*, 1267–1274.

Jennings, V.M., Actor, J.K., Lal, A.A., and Hunter, R.L. (1997). Cytokine profile suggesting that murine cerebral malaria is an encephalitis. Infect. Immun. *65*, 4883–4887.

Karlhofer, F.M., and Yokoyama, W.M. (1991). Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen. IL-2-activated NK cells possess additional specific stimulation pathways. J. Immunol. *146*, 3662–3673.

Kawakami, K., Kinjo, Y., Uezu, K., Yara, S., Miyagi, K., Koguchi, Y., Nakayama, T., Taniguchi, M., and Saito, A. (2001). Monocyte chemoattractant protein-1-dependent increase of V alpha 14 NKT cells in lungs and their roles in Th1 response and host defense in cryptococcal infection. J. Immunol. *167*, 6525–6532.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., et al. (1997). CD1d-restricted and TCR-mediated activation of V_α14 NKT cells by glycosylceramides. Science *278*, 1626–1629.

Langhorne, J., Gillard, S., Simon, B., Slade, S., and Eichmann, K. (1989). Frequencies of CD4$^+$ T cells with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with T_{h1} and T_{h2} characteristics during infection. Int. Immunol. *1*, 416–424.

Lanier, L.L. (1998). NK cell receptors. Annu. Rev. Immunol. *16*, 359–393.

Lehuen, A., Lantz, O., Beaudoin, L., Laloux, V., Carnaud, C., Bendelac, A., Bach, J.F., and Monteiro, R.C. (1998). Overexpression of natural killer T cells protects Vα14-Jα281 transgenic nonobese diabetic mice against diabetes. J. Exp. Med. *188*, 1831–1839.

Lucas, R., Juillard, P., Decoster, E., Redard, M., Burger, D., Donati, Y., Giroud, C., Monso-Hinard, C., De Kesel, T., Buurman, W.A., et al. (1997). Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. Eur. J. Immunol. *27*, 1719–1725.

Mason, L.H., Willette-Brown, J., Mason, A.T., McVicar, D., and Ortaldo, J.R. (2000). Interaction of Ly-49D+ NK cells with H-2Dd target cells leads to Dap-12 phosphorylation and IFN-gamma secretion. J. Immunol. *164*, 603–611.

Miller, L.H., Baruch, D.I., Marsh, K., and Doumbo, O.K. (2002). The pathogenic basis of malaria. Nature *415*, 673–679.

Molano, A., Park, S.H., Chiu, Y.H., Nosseir, S., Bendelac, A., and Tsuji, M. (2000). Cutting edge: the IgG response to the circumsporozoite protein is MHC class II-dependent and CD1d-independent: exploring the role of GPIs in NK T cell activation and antimalarial responses. J. Immunol. *164*, 5005–5009.

Naiki, Y., Nishimura, H., Kawano, T., Tanaka, Y., Itohara, S., Taniguchi, M., and Yoshikai, Y. (1999). Regulatory role of peritoneal NK1.1+ alpha beta T cells in IL-12 production during Salmonella infection. J. Immunol. *163*, 2057–2063.

Porcelli, S.A. (1995). The CD1 family: a third lineage of antigen-presenting molecules. Adv. Immunol. *59*, 1–98.

Rudin, W., Eugster, H.P., Bordmann, G., Bonato, J., Muller, M., Yamage, M., and Ryffel, B. (1997a). Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. Am. J. Pathol. *150*, 257–266.

Rudin, W., Favre, N., Bordmann, G., and Ryffel, B. (1997b). Interferon-gamma is essential for the development of cerebral malaria. Eur. J. Immunol. *27*, 810–815.

Scalzo, A.A., Lyons, P.A., Fitzgerald, N.A., Forbes, C.A., and Shellam, G.R. (1995). The BALB.B6-*Cmv1^r* mouse: a strain congenic for Cmv1 and the NK gene complex. Immunogenetics *41*, 148–151.

Scalzo, A.A., Brown, M.G., Chu, D.T., Heusel, J.W., Yokoyama, W.M., and Forbes, C.A. (1999). Development of intra-natural killer complex (NKC) recombinant and congenic mouse strains for mapping and functional analysis of NK cell regulatory loci. Immunogenetics *49*, 238–241.

Schofield, L., McConville, M.J., Hansen, D., Campbell, A.S., Fraser-Reid, B., Grusby, M.J., and Tachado, S.D. (1999). CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. Science *283*, 225–229.

Scott, P., Pearce, E., Cheever, A.W., Coffman, R.L., and Sher, A. (1989). Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. Immunol. Rev., 161–182.

Shi, F.-D., Flodstrom, M., Balasa, B., Kim, S.H., Van Gunst, K., Strominger, J.L., Wilson, B., and Sarvetnick, N. (2001). Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. Proc. Natl. Acad. Sci. USA *98*, 6777–6782.

Singh, N., Hong, S., Scherer, D.C., Serizawa, I., Burdin, N., Kronenberg, M., Koezuka, Y., and Van Kaer, L. (1999). Cutting edge: activation of NK T cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. J. Immunol. *163*, 2373–2377.

Smiley, S.T., Kaplan, M.H., and Grusby, M.J. (1997). Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science *275*, 977–979.

Smyth, M.J., Crowe, N.Y., and Godfrey, D.I. (2001). NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int. Immunol. *13*, 459–463.

Sonoda, K.H., Exley, M., Snapper, S., Balk, S.P., and Stein-Streilein, J. (1999). CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. J. Exp. Med. *190*, 1197–1200.

Taylor-Robinson, A.W., Phillips, R.S., Severn, A., Moncada, S., and Liew, F.Y. (1993). The role of T_H1 and T_H2 cells in a rodent malaria infection. Science *260*, 1931–1934.

White, N.J., and Ho, M. (1992). The pathophysiology of malaria. Adv. Parasitol. *31*, 83–173.

World Health Organization. (1992). World malaria situation 1990. Division of Control of Tropical Diseases. World Health Stat. Q. *45*, 257–266.

Wilson, S.B., Kent, S.C., Patton, K.T., Orban, T., Jackson, R.A., Exley, M., Porcelli, S., Schatz, D.A., Atkinson, M.A., Balk, S.P., et al. (1998). Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. Nature *391*, 177–181.

Wunderlich, F., Mossmann, H., Helwig, M., and Schillinger, G. (1988). Resistance to *Plasmodium chabaudi* in B10 mice: influence of the H-2 complex and testosterone. Infect. Immun. *56*, 2400–2406.

Yoshimoto, T., and Paul, W.E. (1994). $CD4^{pos}$, $NK1.1^{pos}$ T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. J. Exp. Med. *179*, 1285–1295.

IMMUNOLOGICAL PROCESSES IN MALARIA PATHOGENESIS

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Abstract | Malaria is possibly the most serious infectious disease of humans, infecting 5–10% of the world's population, with 300–600 million clinical cases and more than 2 million deaths annually. Adaptive immune responses in the host limit the clinical impact of infection and provide partial, but incomplete, protection against pathogen replication; however, these complex immunological reactions can contribute to disease and fatalities. So, appropriate regulation of immune responses to malaria lies at the heart of the host–parasite balance and has consequences for global public health. This Review article addresses the innate and adaptive immune mechanisms elicited during malaria that either cause or prevent disease and fatalities, and it considers the implications for vaccine design.

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Malaria is transmitted to vertebrate hosts, such as mice, monkeys and humans, by the bite of female *Anopheles* mosquitoes that are infected with protozoan parasites of the genus *Plasmodium*. The inoculated sporozoite stage is transient and causes no pathology. Within a few minutes, it infects liver cells and undergoes a period of intracellular replication, which is also clinically silent. After liver-stage replication is complete, the parasite initiates blood-stage infection, which is the main cause of disease (FIG. 1). There are four *Plasmodium* species that infect humans. *Plasmodium ovale* typically causes a relatively benign infection. *Plasmodium malariae* is also frequently clinically silent, although an immune-complex-associated glomerulonephropathy can develop following chronic infection. Although it is rarely fatal, *Plasmodium vivax* is a common cause of acute febrile illness, especially in Asia, South America and Oceania, and it might contribute to anaemia. However, most cases of severe disease and most deaths are caused by the blood-stage cycle of *Plasmodium falciparum*, which is endemic in most of sub-Saharan Africa and throughout most of the tropics.

Worldwide, most infections with malaria-causing agents are clinically silent, reflecting the ability of adaptive immune mechanisms to prevent disease. In non-immune individuals, however, infections are more clinically overt, and a minority of these can become severe or life threatening, manifesting a range of discrete and overlapping disease syndromes of complex aetiologies. Those dying of malaria can have single-organ, multiple-organ or systemic involvement (TABLE 1). Overall patterns of disease depend markedly on the age and the previous immunological experience of the host[1]. In areas of high malaria transmission, the burden of disease is borne by infants and young children; life-threatening disease in this setting typically consists of metabolic acidosis (which leads to respiratory distress), cerebral malaria (CM) and severe malarial anaemia (SMA). However, in areas of lower transmission, primary infections might occur in adulthood, in which severe disease more frequently involves additional disturbances, such as renal failure, pulmonary oedema, shock and jaundice. So, transmission dynamics and host age are important determinants of disease, together with host genetics and immunological responses (discussed later).

The diversity of syndromes (TABLE 1) seems to confound the identification of unifying mechanisms of disease. However, the studies reviewed here generally support a scheme in which several important malaria syndromes might arise from the intersection of a few basic processes: the site-specific localization of parasitized red blood cells (PRBCs) among target organs;

the local and systemic action of bioactive parasite products, such as toxins, on host tissues; the local and systemic production of pro-inflammatory and counter-regulatory cytokines and chemokines by the innate and adaptive immune systems in response to parasite products; and the activation, recruitment and infiltration of inflammatory cells. According to this view, diverse organ-specific or systemic disease syndromes are end-stage processes of atypical inflammatory cascades that are initiated in target organs by pathogen products and are maintained by infiltrating cells through positive-feedback cycles. In most cases, homeostasis corrects the cascade effect, and responses are adequately downregulated. In severe disease, however, a 'run-away' effect can ensue, with fatal consequences. Appropriate regulation of immune responses might therefore be a key to healthy outcomes, and understanding these processes might aid in the development of vaccine-based interventions.

Initiation of malaria-associated syndromes

Site-specific localization of PRBCs. As blood-stage parasites mature through the 48-hour replicative cycle, avoiding passage through the spleen is an essential survival strategy, because this immunological effector organ[2] efficiently filters PRBCs from the bloodstream. Erythrocyte membrane protein 1 (EMP1) is the name given collectively to members of a family of variant cell-surface proteins that are encoded by *P. falciparum* and enable PRBCs to engage multiple receptors — such as intercellular adhesion molecule 1 (ICAM1), vascular cell-adhesion molecule 1 (VCAM1), CD31, CD36,

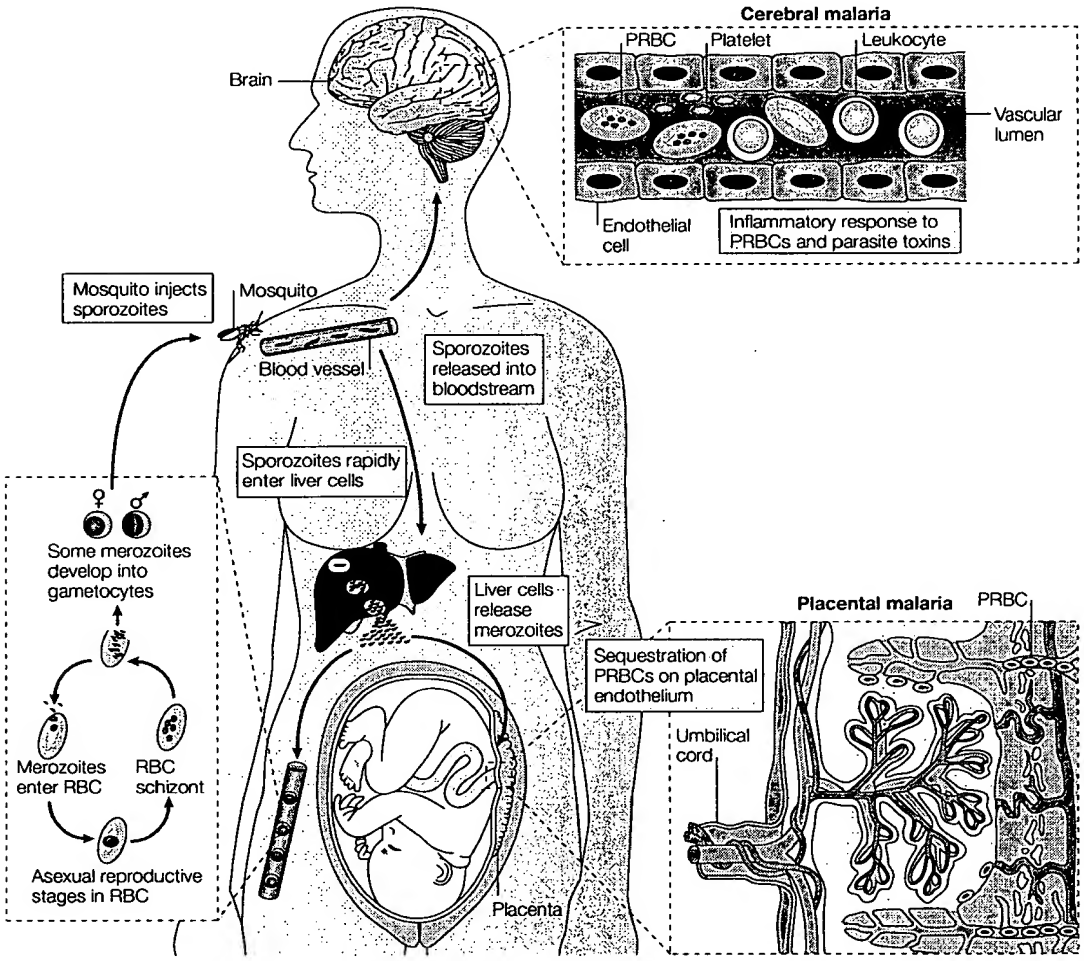


Figure 1 | **The life cycle of *Plasmodium falciparum*.** Mosquitoes that carry the malaria-causing parasite *Plasmodium falciparum* inject a small number of infectious sporozoites into the bloodstream while feeding. Within a few minutes, they are carried to the liver, where they invade and replicate in liver cells. Then, 10–12 days later, thousands of daughter merozoites are released back into the bloodstream and enter red blood cells (RBCs). The parasites are carried around the circulation within RBCs, but as they grow, they express adherent ligands — such as *P. falciparum* erythrocyte membrane protein 1 — that enable the maturing parasite to bind receptors expressed by endothelial cells that line the blood vessels in the deep vascular beds of organs such as the brain, lungs and placenta. After 48 hours, the parasitized RBCs (PRBCs) rupture and release more daughter merozoites, thereby perpetuating and promoting the blood-stage cycle. The presence of the parasite and the invasion of RBCs might not be sufficient to account for disease; instead, the release of bioactive parasite molecules and an inappropriately regulated host immune response could be the main causes of fatal pathogenesis, which occurs in only a minority of patients. Some merozoites differentiate into gametocytes, which, when taken up by another feeding mosquito, perpetuate the sexual cycle in the insect.

CHAGAS' DISEASE
A disease that is caused by *Trypanosoma cruzi*. In chronic cases, it is associated with autoimmune damage to various organs.

thrombospondin, endothelial-cell selectin (E-selectin), chondroitin sulphate A (CSA) and hyaluronic acid — that are expressed by vascular endothelial cells in deep-organ microvascular beds. Binding to these receptors by cell-surface *P. falciparum* EMP1 sequesters parasites so that they are removed from the circulation and, consequently, do not travel to the spleen. Although this is advantageous for the survival of the parasite, this strategy has the pathological consequence of concentrating parasites in various target organs, and the precise locations depend on the differential expression of the various *P. falciparum* EMP1 members and their diverse endothelial-cell-expressed receptors.

Production of bioactive parasite products. As sequestered parasites mature, they produce a variety of bioactive molecules that either upregulate or downregulate pathogenic processes, largely through their effects on the innate immune system (TABLE 2). Immune responses to infectious insults are mainly initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with receptors expressed by host cells. For viruses, bacteria and yeast, PAMPs include modified lipids (such as bacterial lipopolysaccharides), carbohydrates (such as yeast zymosan), proteins (such as flagellin) and nucleic acids (such as unmethylated CpG-motif-containing DNA and double-stranded RNA). Many studies implicate glycosylphosphatidylinositol (GPI) of *P. falciparum* as a malaria PAMP and as a toxin. Purified GPI induces the expression of many genes that are implicated in malaria pathogenesis: for example, genes that encode pro-inflammatory cytokines — such as tumour-necrosis factor (TNF), interleukin-1 (IL-1) and IL-12 (REFS 3–6) — inducible nitric-oxide synthase[7], and various adhesion molecules that are expressed at the surface of the vascular endothelium and are recognized by *P. falciparum* EMP1 (REF. 8), which increases endothelial-cell binding by PRBCs[8]. In a sepsis–shock model, GPI alone is sufficient to cause symptoms that are similar to those of acute malaria, such as transient pyrexia, hypoglycaemia and death of recipients as a consequence of TNF-mediated coagulopathy, as seen in the malarial shock-like syndrome[3] (TABLE 1). The GPIs from *Trypanosoma brucei*, *Trypanosoma cruzi* and *Toxoplasma gondii* all have similar properties to the GPI from *P. falciparum*[9–11], and this might account for some pathogenic features of trypanosomiasis, CHAGAS' DISEASE and toxoplasmosis.

Other potential *P. falciparum* PAMPs include phosphorylated, non-peptidic antigens, to which γδ T cells respond with slow kinetics[12], and haemozoin, the insoluble, crystalline residue of parasite-mediated haemoglobin digestion, which is long-lived and accumulates in phagocytes. Haemozoin has interesting, although seemingly contradictory, bioactivities. It has been reported to induce[13] or inhibit[14] dendritic cell (DC) maturation and to induce either the production of the T helper 1 (T_H1) cytokines TNF[15] and IL-12 (REF. 13) or the T_H2 cytokine IL-10. It also inhibits general proliferative responses by human leukocytes[16]. In addition, it has been shown to promote monocyte and macrophage dysfunction, by impairing phagocytosis and the expression of MHC class II molecules, CD11c and ICAM1 (REF. 17). Overall, haemozoin seems to be highly immunosuppressive[18,19].

Table 1 | **Severe and fatal disease syndromes in malaria**

Syndrome	Clinical features	Possible sequence or mechanism of disease
Cerebral malaria	Sustained impaired consciousness, coma, long-term neurological sequelae	Cerebral parasite sequestration; bioactive GPI; pro-inflammatory cytokine cascade; endothelial-cell activation; natural killer T-cell activation; T_H1/T_H2-cell balance; chemokine production; monocyte, macrophage and neutrophil recruitment; platelet and fibrinogen deposition; $CD4^+$, $CD8^+$ and γδ T-cell involvement; IFN-γ production; neurological metabolic derangements; possibly hypoxia
Placental malaria	Placental insufficiency, low birth weight, premature delivery, loss of fetus	*Plasmodium falciparum* EMP1-mediated binding to placental endothelium and syncytiotrophoblast through chondroitin sulphate A and hyaluronic acid; cytokine production; chemokine-mediated recruitment and infiltration of monocytes; intravascular macrophage differentiation
Severe malarial anaemia	Pallor, lethargy, haemoglobin level of 4–6 g per 10 ml	Erythropoietic suppression by toxins and cytokines; increased RBC destruction, owing to parasitization, RBC alterations, complement and immune complex or antigen deposition, erythrophagocytosis, splenic hyperphagism, $CD4^+$ T cells, T_H1/T_H2 cytokine balance (TNF and IFN-γ versus IL-10)
Metabolic acidosis	Respiratory distress, deep breathing (Kussmaul breathing), hypovolaemia	Molecular mechanisms unknown. Possibly widespread parasite sequestration; bioactive toxins; increased vascular permeability; reduced tissue perfusion; anaemia; pulmonary airway obstruction; hypoxia; increased host glycolysis; repressed gluconeogenesis. Some overlap with shock-like syndrome
Shock-like syndrome (systemic inflammatory-response-like syndrome)	Shock, haemodynamic changes, impaired organ perfusion, disseminated intravascular coagulation	Bioactive toxins; T_H1 cytokines; acute-phase reactants

EMP1, erythrocyte membrane protein 1; GPI, glycosylphosphatidylinositol; IFN-γ, interferon-γ; IL-10, interleukin-10; RBC, red blood cell; T_H, T helper; TNF, tumour-necrosis factor.

ERYTHROPOIETIC SUPPRESSION
The inhibition of normal production of fresh red blood cells in the bone marrow or spleen. This occurs by various mechanisms, including inhibition of precursor-cell responsiveness to erythropoietin.

Table 2 | **Malaria products and their bioactivities**

Parasite product	Receptor and cell type	Pathological and cellular effects
Plasmodium falciparum EMP1-family members	ICAM1, VCAM1, CD36, thrombospondin, E-selectin, chondroitin sulphate A, hyaluronic acid and CD31 on endothelial cells and trophoblast cells; CD36 on DCs	Binding directs parasite to the brain, placenta and possibly other target organs; CD36 engagement proposed to suppress DC and macrophage activation
GPI	TLR2, TLR4 and/or possibly C-type lectins on several cell types, including DCs, macrophages, endothelial cells and adipocytes; CD1d and Vα14–Vβ8 TCR on NKT cells	Induces widespread expression of genes encoding pro-inflammatory proteins (including TNF, IL-1, IL-6, IL-12, iNOS, ICAM1, VCAM1); activates NKT cells; induces T_H1- or T_H2-cytokine production
Haemozoin	TLR9 on DCs	Contradictory reports: both T_H1- and T_H2-cell activities; induces and inhibits DCs; suppresses macrophages; induces IL-10 production; broadly immunosuppressive
Unknown ligands	NKC-encoded receptors on NK and NKT cells	Activates NK cells; induces IFN-γ production; regulates balance of T_H1 and T_H2 cytokines produced by NKT cells
Isopentenyl pyrophosphate	γδ TCRs	Activates γδ T cells; induces IFN-γ production
Protein antigens	Diverse TCRs on $CD4^+$ and $CD8^+$ T cells	Activates αβ T cells; induces T_H1- or T_H2-cytokine production
Unknown sugar(s)	MBL in plasma	Possible binding provides protection; low levels of MBL are associated with disease

DC, dendritic cell; EMP1, erythrocyte membrane protein 1; E-selectin, endothelial-cell selectin; GPI, glycosylphosphatidylinositol; ICAM1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric-oxide synthase; MBL, mannose-binding lectin; NK, natural killer; NKC, natural killer complex; NKT, natural killer T; TCR, T-cell receptor; T_H, T helper; TLR, Toll-like receptor; TNF, tumour-necrosis factor; VCAM1, vascular cell-adhesion molecule 1.

Divergent results might be a consequence of differences in haemozoin preparations, because these preparations are heterogeneous, containing uncharacterized bioactive contaminants, such as non-covalently associated phospholipids, hydroxylated fatty acids, carbohydrates and glycolipids. Removal of non-covalently associated lipids from haemozoin preparations abolishes their bioactivity[20,21]. However, a synthetic version of haemozoin, β-haematin, has pro-inflammatory activity on mouse monocytes and macrophages[13,22].

At high PRBC to target-cell ratios, PRBCs can inhibit the maturation of DCs and reduce their ability to stimulate T cells[23,24]. DC exhaustion could result from antigen overload, but it has been suggested, although not proven, that these activities result from the binding of *P. falciparum* EMP1 to CD36 and CD51 (REFS 23,24). If this is the case, this important molecule would also participate in the negative regulation of the immune system, in contrast to PAMPs, which only induce activation of the innate immune system.

Recognition of parasite molecules by innate immune receptors. The various members of the mammalian Toll-like receptor (TLR) family are important receptors that are responsible for recognition of microbial PAMPs. Mice that have a mutant version of TLR4, which binds lipopolysaccharide, are responsive to GPI from *P. falciparum*[3] and *T. cruzi*, indicating that this glycolipid triggers a different receptor that results in the expression of pro-inflammatory genes. GPI from both pathogens can activate TLR2, and this requires the crucial TLR adaptor protein MyD88 (myeloid differentiation primary-response gene 88)[25,26]. However, TLR2-deficient mice produced pro-inflammatory cytokines when stimulated with live *T. cruzi*, and they were almost as resistant to infection as wild-type mice[27]. Evidence for the involvement of TLR mechanisms in the immunoregulation and immunopathogenesis of malaria is still limited. MyD88-deficient mice that are infected with *Plasmodium berghei* have less liver injury and produce less IL-12 but not IL-18 than wild-type mice[28]. However, the liver injury in this experimental system does not model the pathophysiological disease processes that occur in humans. Haemozoin activates mouse DCs through TLR9 (REF. 13). TLR9 is expressed by monocytes, macrophages, B cells and DCs in mice, but it is restricted to B cells and plasmacytoid DCs in humans[29]. So, TLR9 agonists (and presumably haemozoin) do not activate monocytes or macrophages in humans. TLR9 functions through a strictly MyD88-dependent pathway. However, data indicate that there is no significant difference in peak parasitaemia, ERYTHROPOIETIC SUPPRESSION, interferon-γ (IFN-γ) production and CM fatality rates between *P. berghei*-infected, *Myd88*$^{-/-}$ mice and their *P. berghei*-infected, *Myd88*$^{+/-}$ litter-mates (L.S., unpublished observations). If this observation is confirmed, then MyD88-independent pathways would seem to dominate disease processes in this model. Other lectin-like receptors might also function as pattern-recognition receptors or modulatory receptors, including calcium-dependent C-type lectins such as soluble mannose-binding lectin (MBL), which is present in the plasma. MBL binds sugars that are present at the surface of PRBCs but have yet to be characterized[30], and a low level of MBL in the

plasma is associated with severe disease in humans[31]. Nonetheless, lectin-like-receptor signalling and bioactivity remain poorly defined. Further studies are clearly required to dissect the role of purified, structurally defined malaria PAMPs in the activation of TLR-signalling pathways, both those that are MyD88 dependent and MyD88 independent, and to elucidate the possible contributions of these pathways to relevant pathophysiological processes.

'Intermediate' pathways between innate and adaptive immunity. Many studies show that pro-inflammatory T_H1 cytokines are crucial determinants of malaria disease states. In addition to acute-phase monokines, the production of which is induced rapidly by parasite toxins, IFN-γ levels can increase very early during malaria, indicating that non-conventional lymphoid populations that can function with accelerated kinetics account for this production. CD1d-restricted natural killer T (NKT) cells are such an 'intermediate' arm between innate and adaptive immunity, and these cells are particularly important in regulating the downstream differentiation of $CD4^+$ T cells into T_H1 and T_H2 cells[32]. As well as having toxic bioactivity, GPI is a natural glycolipid ligand for NKT cells[33], together with closely related mycobacterial phosphatidylinositol mannosides[34]. In mice with CM, NKT cells were shown to be a crucial determinant of cytokine levels, the pro-inflammatory cascade, pathogenesis and fatality[35]. The CD1d–NKT-cell pathway also upregulates and downregulates acute malarial splenomegaly in mice and is an important determinant of B-cell responses[36]. The CD1d–NKT-cell pathway either prevents or promotes fatality, and it determines the differentiation of immune cells, depending on which alleles of the natural killer complex (NKC), which is located on mouse chromosome 6 (REF. 37), are expressed. The loci in the NKC are differentially expressed by natural killer (NK) cells and NKT cells, and they control the production of pro-inflammatory T_H1 cytokines and counter-regulatory T_H2 cytokines by NKT cells[35]. NKC loci also determine the level of malarial anaemia, the isotypes of malaria-specific antibodies and the T_H1/T_H2 profile of conventional T cells that is induced during infection[37]. C57BL/6 mice, which are T_H1-cell-response prone, are susceptible to CM, whereas BALB/c mice, which have a genetically determined T_H2-cell bias, are resistant[38], and these different profiles reflect the substantial contribution of polymorphic NKC loci[37]. Infection with malaria-causing agents imparts NKC-dependent signals to NKT cells that influence their differentiation into cells that secrete T_H1 or T_H2 cytokines, but the specific receptor–ligand interactions that are involved in this process are unknown. So, the NKC is a crucial genetic determinant of malaria pathogenesis in mice, with an important role in controlling NK- and NKT-cell function. Not all NKC loci are involved, however, because injecting certain NK-cell-specific monoclonal antibodies does not affect pathogenesis in mice[39]. Human NK cells also become activated early during malaria[40] and are activated rapidly by parasites *in vitro*[41], which requires direct contact of PRBCs with NK cells and results in IFN-γ production[42]. However, the relevance of NK cells to human disease remains unclear.

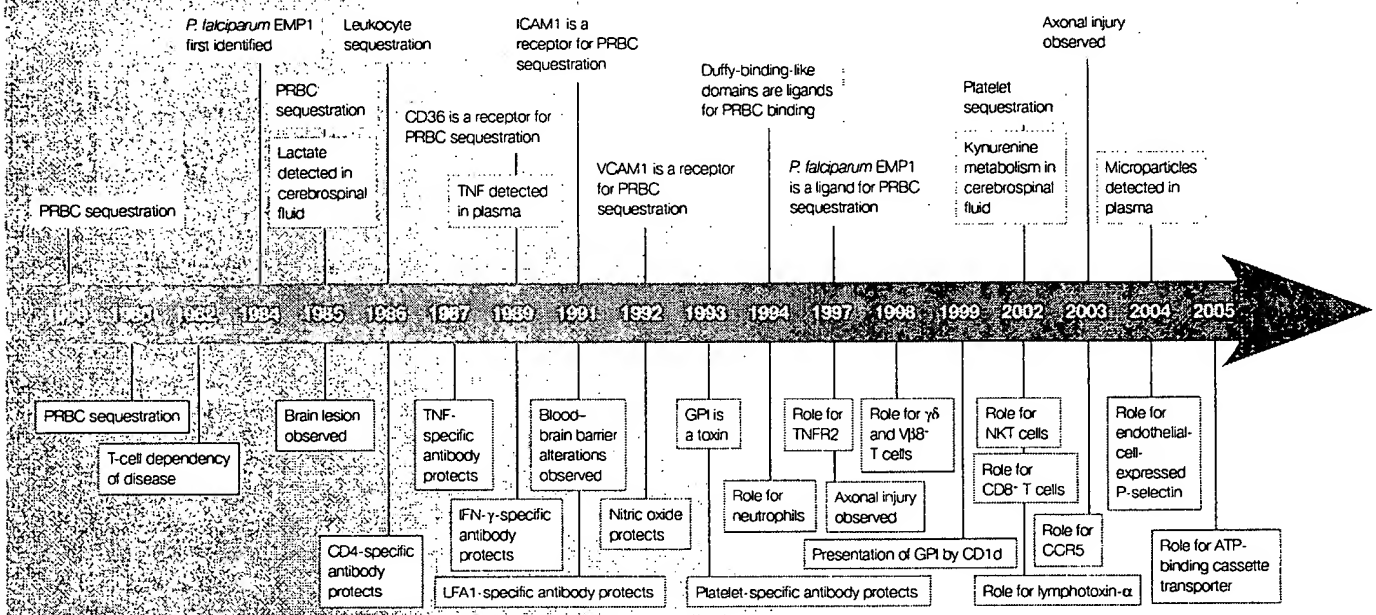
Progression to cerebral malaria

The histopathology of CM is associated with the accumulation of mature PRBCs in cerebral microvessels, through sequestration. This feature was first described in 1894 (REF. 43) and has since been confirmed by numerous studies[44]. This has led to the dominant theory of CM pathogenesis: that, because PRBCs are sequestered in brain capillaries and post-capillary venules, they induce flow perturbations that eventually lead to obstruction and hypoxia of the surrounding brain parenchyma[45,46] and to haemorrhages. Nevertheless, as early as 1944, there were doubts about any causal relationship between CM and PRBC sequestration[44]. Indeed, there is, for the most part, no proof that PRBC sequestration is sufficient to cause CM[47] and even less that it is a cause of death; this issue has been debated previously[48,49]. Mature-stage parasites are absent from the peripheral blood of patients infected with *P. falciparum*. Clearly, sequestration of PRBCs in deep microvascular beds occurs routinely in all of these patients, although only 1% of these individuals develop CM. So, PRBC sequestration might not be sufficient to cause CM, but it might be necessary. Subsequently, it has become apparent in humans with CM, as well as in mouse models of CM, that host cells, such as leukocytes or platelets, might also be sequestered in brain microvessels, in addition to PRBCs[50,51]. These host cells might be involved in the pathogenesis of CM, either through local effects in brain microvessels or through distant effects mediated by the production of potentially deleterious mediators, such as pro-inflammatory cytokines, which can be detected in the circulation. These leukocytes, however, show little evidence of endothelial extravasation and therefore cannot be described as classic inflammatory cells. The TIMELINE shows how the study of mouse and simian models, *in vitro* assays and human infections has contributed over time to the elucidation of the complex cascade that controls CM pathogenesis.

Accumulation of intravascular infiltrates. The intravascular accumulation of monocytes in the brain has long been recognized in mice[52–54] and humans[51,55–60] with CM. Sequestered monocytes and macrophages are more abundant in paediatric patients with CM than in those with SMA or non-malarial encephalopathy[44]. This feature has largely been ignored as a possible contributor to the pathogenic process in CM in humans.

In CM, some cells of the monocyte–macrophage lineage within the microvasculature show characteristics that are normally associated with tissue macrophages (including an increased size and the presence of phagocytosed material, vacuoles, a ruffled plasma membrane and pseudopods). For this reason, we refer to these cells as intravascular macrophages. Therefore, CM is a rare situation in which monocytes

Timeline | **Contribution of animal models and human-based assays to our understanding of cerebral malaria**



Research on cerebral malaria has been carried out in mouse *in vivo* infection models (orange), simian *in vivo* infection models (green), *in vitro* models of human infection (yellow) and *in vitro* assays involving human tissues (blue). CCR5, CC-chemokine receptor 5; EMP1, erythrocyte membrane protein 1 of *P. falciparum*; E-selectin, endothelial-cell selectin; GPI, glycosylphosphatidylinositol; ICAM1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; LFA1, lymphocyte function-associated antigen 1; NKT, natural killer T; *P. falciparum*, *Plasmodium falciparum*; PRBC, parasitized red blood cell; P-selectin, platelet selectin; TNF, tumour-necrosis factor; TNFR2, TNF receptor 2; VCAM1, vascular cell-adhesion molecule 1.

differentiate into macrophages in the intravascular compartment and not in the tissues. Several modifications of monocyte–macrophage phenotype and functions are known to occur in severe malaria. For example, coagulation factors secreted by monocytes, in addition to their role in blood-clot formation, might contribute to the sequestration of cells in brain microvessels of patients with CM, at least in children. Tissue factor can itself function as an adhesion molecule, and it has coagulation-independent roles in cell adhesion and migration. Furthermore, upregulation of tissue-factor expression has a central role in driving a thrombosis–inflammation circuit. Coagulant mediators (such as factor VIIa and factor Xa) and the end-product fibrin are also pro-inflammatory, eliciting the expression of TNF and other cytokines, as well as chemokines and adhesion molecules, and this has recently been reviewed in REFS 50,51.

The accumulation of leukocytes in the brains of patients with CM is evidence of a considerable chemokine cascade, which has been shown in experimental studies, including DNA-microarray analyses of mouse disease[61,62]. The expression of monocyte-secreted cytokines and chemokines, such as TNF, CXC-chemokine ligand 10 (CXCL10; also known as IP10), CC-chemokine ligand 2 (CCL2; also known as MCP1) and CCL5 (also known as RANTES), varies with mouse genotype and correlates with resistance versus susceptibility to disease[62]. Neutrophils also contribute to the brain lesions in mice and are an important source of cytokines (including the p40 subunit of IL-12, IL-18, IFN-γ and TNF) and chemokines (including CCL3 (also known as MIP1α), CXCL9 (also known as MIG) and CXCL10) that participate in pathogenesis. Indeed, depletion of neutrophils early in malaria prevents the development of CM in mice, downregulates the expression of T_H1 cytokines in the brain, and markedly decreases the sequestration of monocytes and the incidence of microhaemorrhages in the brain[63]. Although the contribution of neutrophils to CM in humans is unknown, the neutrophil-specific activation marker and recruitment agent lipocalin was found at a higher concentration in plasma from patients with severe malaria[64], and transcription of the gene encoding lipocalin is upregulated in the brain during cerebral disease in mice[61].

Evidence for a pathogenic role of platelets, both in CM in mice and in *in vitro* models of CM in humans, has been summarized elsewhere[65]. As illustrated in FIG. 2, there are several possible ways through which platelets could affect endothelial-cell function and viability, and promote leukocyte adhesion. First, platelets, together with other cell types, can modulate the expression of adhesion molecules, such as ICAM1, and the production of cytokines, such as IL-6, by endothelial cells[66], through the release of IL-1. Second, platelet-derived microparticles modulate endothelial-cell metabolism, by regulating the production of cyclooxygenase-2 and prostaglandins[67], and increase the adhesiveness of the endothelial-cell–leukocyte–platelet interaction,

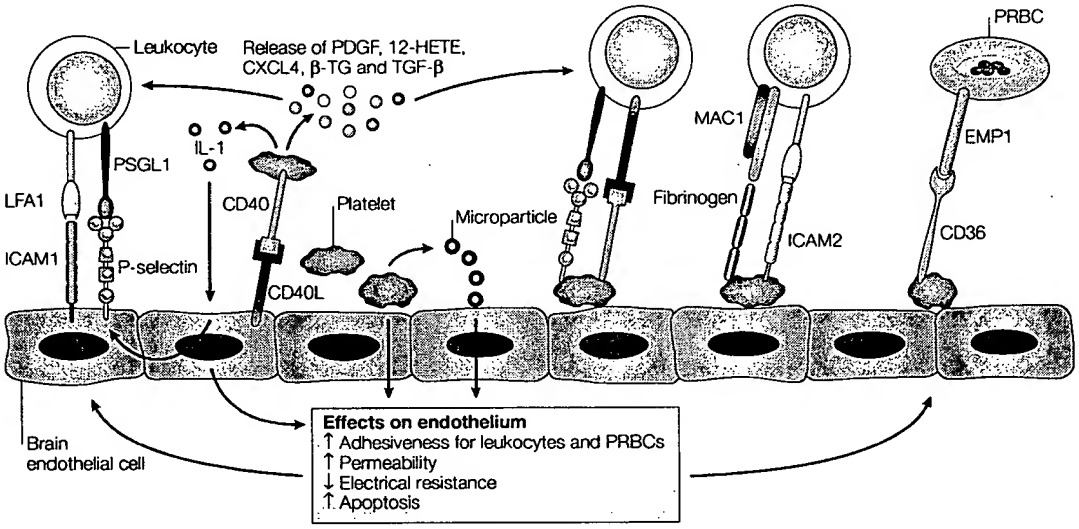


Figure 2 | **Mechanisms of platelet–endothelial-cell interactions in cerebral malaria.** Platelets, after activation by tumour-necrosis factor, can markedly alter the functions of brain endothelial cells, either directly, by binding to the endothelium, or indirectly, by releasing molecules from their secretory granules. For example, platelets release interleukin-1 (IL-1), which increases the expression of adhesion molecules (such as intercellular adhesion molecule 1, ICAM1) and the production of cytokines (such as IL-6) by endothelial cells. Platelet-derived microparticles also alter endothelial-cell metabolism by regulating the production of cyclooxygenase-2 and prostaglandins, which might affect endothelium permeability, electrical resistance and apoptosis. One of the earliest events in inflammation or tissue injury is the adhesion of activated platelets to the endothelium (possibly through CD40 ligand (CD40L)–CD40 interactions). Importantly, in cerebral malaria, this provides additional receptors at the endothelial-cell surface for the adhesion of leukocytes, which bind platelet surface molecules such as platelet selectin (P-selectin) and CD40L, through P-selectin glycoprotein ligand 1 (PSGL1) and CD40, respectively. Furthermore, platelet-derived microparticles can increase the adhesiveness of endothelial cells to leukocytes and the adhesiveness of the platelets themselves to the endothelium. Finally, activated platelets can indirectly alter endothelial cells through the complex effects of their granule-derived mediators on leukocytes, including platelet-derived growth factor (PDGF), 12-hydroxyeicosatetraenoic acid (12-HETE), CXC-chemokine ligand 4 (CXCL4; also known as PF4), β-thromboglobulin (β-TG) and transforming growth factor-β (TGF-β). The exact mechanisms that are involved have yet to be defined. EMP1, erythrocyte membrane protein 1 of *Plasmodium falciparum*; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage receptor 1; PRBC, parasitized red blood cell.

by directly upregulating the expression of adhesion molecules and integrins such as ICAM1 and macrophage receptor 1 (MAC1; CD11b–CD18), both by the endothelium and by adherent monocytes and other leukocytes[68]. Consistent with this, microparticles have been shown to be crucial for the development of CM in mice[69], and the level of microparticles is markedly increased in the plasma of children with CM[70]. Third, the adhesion of activated platelets to the endothelium (which is an early event in inflammation and possibly occurs through CD40-ligand–CD40 interactions) can indirectly mediate leukocyte–endothelial-cell adhesion, by providing additional receptors, such as fibrinogen and ICAM2 (REF. 71), which are ligands for leukocyte MAC1 and lymphocyte function-associated antigen 1 (LFA1; CD11a–CD18), respectively. In addition, platelet selectin (P-selectin) is released from secretory granules of activated platelets and can be expressed at their surface, and leukocytes that express P-selectin glycoprotein ligand 1 can then adhere to this surface P-selectin[72]. Last, platelets can also modulate the sequestration of normal RBCs and of PRBCs, by surface expression of CD36, which binds *P. falciparum* EMP1 at the PRBC surface, and they can directly modulate cytokine production by circulating leukocytes and the endothelium, as has been shown in mouse models of CM[73].

A key unresolved question is how much of the accumulation of parasites in cerebral vessels is associated with adhesion mediated by classic receptor–ligand interactions and how much is associated with non-specific deposition of activated platelets, deposition of fibrin and the presence of other markers of the host inflammatory response. Evidence from P-selectin- or ICAM1-deficient mice indicates that most platelet binding is likely to be ligand mediated (not non-specific), because platelet binding is undetectable by intravital microscopy in these mutant mice[74]. Nonetheless, the relative importance of the numerous effects of platelets in the pathogenesis of CM remains to be further elucidated[50].

Role of T cells. In mice, CM has been known to be a T-cell-dependent disease for two decades. NUDE MICE and mice that are deficient in the αβ-TCR are resistant to disease. In addition, *in vivo* depletion using specific monoclonal antibodies showed that $CD4^+$ T cells are required for pathogenesis[75]. However, MHC-class-II-deficient mice, which lack conventional $CD4^+$ T cells, still develop CM[39], probably by retaining CD1d-restricted, $CD4^+$ NKT cells that have a crucial role in disease[35]. Subsequently, γδ T cells have been shown to participate in CM, because although mice that are deficient in the γδ-TCR are susceptible to CM, depletion

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Mice with a mutation that causes both hairlessness and defective formation of the thymus, which results in a lack of mature T cells.

of γδ T cells from wild-type mice (using specific antibodies) confers resistance to CM. γδ-TCR-deficient animals therefore seem to develop a functional redundancy that is absent in conditions of normal immunological ontogeny[76]. In humans, there are few data that address T-cell involvement in CM.

IFN-γ seems to be the most important T-cell-secreted cytokine. *In vivo* neutralization of IFN-γ in mice infected with *P. berghei* strain ANKA prevented TNF overproduction and CM development[77]. The central role of IFN-γ in the pathogenesis of CM was confirmed by experiments in mice that were deficient in IFN-γ or the IFN-γ receptor, which are resistant to the development of experimentally induced CM[78]. The cell-surface antigen CTLA4 (cytotoxic T-lymphocyte antigen 4; CD152) negatively regulates T cells and therefore might downregulate T-cell responses and prevent immunopathology. As expected, blockade of CTLA4 exacerbated CM in mice[79], which highlights the contribution of T cells to disease.

$CD8^+$ T cells might also be effectors in pathogenesis[39,80,81]. In mice, the number of cytotoxic $CD8^+$ T cells infiltrating the brain during CM is increased, and these cells contribute to permeability changes of the mouse blood–brain barrier through perforin-dependent mechanisms[82,83]. CM is also associated with an increase in the number of peripheral $CD8^+$ T cells that have TCRs using the Vβ8.1 or Vβ8.2 segments[84], and disease was reduced when mice were treated with antibodies that specifically neutralize these T cells. Because of the hypothesis that $CD8^+$ T cells contribute to the pathology of CM[39], the role of CC-chemokine receptor 2 (CCR2), which is expressed by $CD8^+$ T cells, was evaluated[85]. Mice that were deficient in CCR2 remained susceptible to CM, indicating that this receptor is not directly involved in cerebral pathology. However, the number of $CD8^+CCR5^+$ T cells in the brain increased after infection with *P. berghei* strain ANKA, and CCR5-deficient mice were partially protected against the neurological syndrome[85]. $CD8^+$ T cells sequestered in the brain were proposed to be responsible for the neurological syndrome and for death, but there has been no report that directly shows the presence of these cells, using histopathology, at the site of neurovascular lesions. Direct contact has been shown *in vitro* between brain endothelial cells and activated T cells, and this contact might also be crucial in cerebral pathogenesis[86]. $CD8^+$ T cells also have a role in circulatory shock and in respiratory distress in mice that are infected with *P. berghei*[87].

Microvascular obstruction: what is the sequence of events? In conclusion, histopathological studies have uncovered the presence of PRBCs, platelets and leukocytes, each of which might contribute to disease, in the cerebral vascular lumen in humans and mice with CM (FIG. 3). What cannot be deduced from such observations is the order in which the cells and platelets are sequestered in a vessel and the nature of the ensuing interactions. There are three possibilities: namely, that PRBCs bind brain endothelial cells first, followed by leukocytes or platelets; that leukocytes bind first; or that platelets make the initial interaction. This might vary in different areas of the brain and over time, thereby leading to a mosaic of arrested cells. So, conclusions drawn from histology, even in post-mortem analyses of multiple sites, might be influenced by sampling variables. In addition, the transient nature of the binding events, the temporal pattern of expression of adhesion molecules, and competition among PRBCs and leukocytes for binding sites is likely to determine the sequence of adhesive events. For example, P-selectin and E-selectin are expressed transiently, which probably influences the binding of circulating cells. Endothelial-cell, leukocyte and platelet adhesion molecules are likely to participate in cell–cell interactions in this dynamic environment. After cells have accumulated, they produce chemotactic, inflammatory and toxic mediators that further contribute to the pathogenesis, and this can lead to positive-feedback cycles (FIG. 3).

Judgement must remain reserved, however, on the contribution of each factor in different age groups, in different transmission settings and across the spectrum of disease. What is cause and what is effect in this complex picture? The difficulty of ascribing function applies particularly to post-mortem histological analyses of human cerebral disease states, because processes that are observed to be associated with disease might be corrective attempts that have been made by the host to control infection or to downregulate pathogenesis, or might be pathogenically neutral. A recent histological study points to the possibility of a disease spectrum in paediatric patients with CM[60]. There are three defined disease categories (CM1, CM2 and CM3), and they occurred in 15%, 56% and 29% of clinically defined cases, respectively. Patients with CM1 have only PRBC sequestration, whereas patients with CM2 have PRBC sequestration plus other intra- and perivascular pathology, including immune-cell infiltrates. Patients with CM3 fulfilled the complete World Health Organization clinical criteria for CM, including unrousable coma associated with infection, but they died of non-malarial causes. So, what seems to be a defined syndrome appears heterogeneous with respect to the underlying pathogenesis[88]. Key histological studies have been undertaken in South-East Asian adults. These implicate a role mainly for PRBC sequestration in CM, and it seems probable that there are substantial differences between CM in adults and in children (studied in Africa), with intravascular leukocyte accumulation being more pronounced in children. Fortunately, animal models allow hypotheses to be tested by experimental intervention, and CM in rodents seems to mirror the human disease with reasonable accuracy, including presenting a disease spectrum that depends on host genetics. Because disease progression in animal models evidently requires several sequential steps, multiple points of intervention might also be possible in the treatment of humans with CM.

Immunology of placental malaria

In areas with high rates of transmission of malaria, women have considerable immunity to malaria by the time they reach child-bearing age. However, during their first or second pregnancy, they are susceptible to placental malaria, and this becomes less common with subsequent pregnancies. CSA and hyaluronic acid are receptors that are expressed by endothelial cells, preferentially in the placenta. PRBCs that display *P. falciparum* EMP1 proteins that bind these receptors can be sequestered, allowing parasite maturation on the placental endothelium[89] and thereby validating the link between an adhesive phenotype and organ-specific disease[90]. As is the case for CM in mice[52,54,91] and humans[51,55,60], severe placental malaria (which results in low birth weight) is associated with substantial macrophage infiltration into the placenta and high chemokine expression[92,93], indicating that adhesive phenotype and chemokine-driven cellular infiltration are key determinants of organ-specific disease syndromes. Interestingly, women with placental malaria remain afebrile, indicating that the condition is mainly a local pathology, with some aspects of acquired clinical immunity remaining unaffected by placental parasitization.

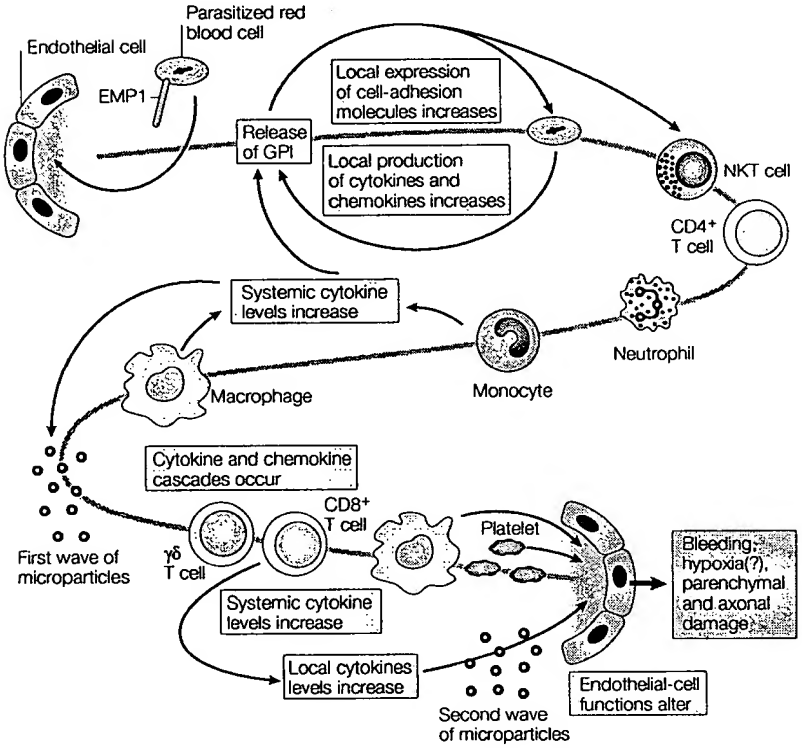

Figure 3 | **Schematic representation of events that are likely to lead to severe malarial disease, particularly in the brain.** First, parasitized red blood cells (PRBCs) adhere to receptors expressed by brain microvascular endothelial cells, such as intercellular adhesion molecule 1 (ICAM1), through surface expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (EMP1). When merozoites are released from PRBCs ~4 hours later, parasite glycosylphosphatidylinositol (GPI), which is either released into the blood or present in parasite membranes, functions as a pathogen-associated molecular pattern and toxin, thereby inducing an inflammatory response. A local acute-phase response then occurs, which involves activation of the endothelium and local production of cytokines and chemokines, and this results in upregulation of expression of cell-adhesion molecules by endothelial cells. Within the next ~24 hours, this cycle is perpetuated and exacerbated, owing to increasing parasite numbers and further binding of PRBCs to endothelial cells that have upregulated expression of cell-adhesion molecules. GPI can also function as a ligand for CD1d-restricted natural killer T (NKT) cells, leading to their activation. Activated NKT cells can regulate the differentiation of $CD4^+$ T cells into T helper 1 (T_H1) or T_H2 cells, depending on which natural-killer-complex loci are expressed, so activation and involvement of $CD4^+$ T cells occurs. In addition, chemokines recruit monocytes and activate neutrophils (although neutrophils are not known to infiltrate brain microvessels in humans or mice with cerebral malaria). Recruited monocytes can then differentiate into macrophages and become arrested in brain microvessels. Macrophages can also be activated by GPI, a process that is amplified by interferon-γ. Local activated macrophages produce more chemokines, which are released systemically, thereby amplifying infiltration of cells, sequestration of PRBCs and release of microparticles (which are probably of endothelial-cell origin). After several more cycles, γδ T cells and $CD8^+$ T cells might become involved, releasing more chemokines and cytokines both systemically and locally and possibly inducing perforin-mediated lesions in the endothelium. Together with locally arrested macrophages, platelets are sequestered and participate in altering endothelial-cell functions. More microparticles of platelet, endothelial-cell and monocyte origin are released, which leads to the dissemination of pro-inflammatory and pro-coagulant effects. Finally, damage to the endothelium, with possible perivascular haemorrhage, axonal injury, and neurotransmitter and metabolic changes, can ensue. The overall disease spectrum in humans might depend on whether all of these processes occur or only some of them.

Immunology of severe malarial anaemia

SMA is the most serious and most common pernicious complication of malaria, and it might be the leading cause of deaths from malaria worldwide. Compared with the florid, acute signs of CM, SMA can be chronic or silent and therefore is less well studied. However, that peaks of SMA incidence coincide with peaks of CM incidence during high transmission seasons in endemic areas indicates that SMA might sometimes be an acute syndrome, notwithstanding that, for some cases, this might be the point at which a chronic process flares up. Many cross-sectional studies report a lack of correlation between parasite densities and severity of anaemia, although longitudinal studies indicate that duration of infection might be a better predictor of risk of disease[94]. There is a misconception, however, that SMA arises simply from the destruction of infected RBCs, and over-reliance on inappropriate models might contribute to this view (BOX 1). Many animals with acute infections develop severe haemolytic anaemia, through invasion and rupture of RBCs as a consequence of excessive parasite burdens of a magnitude that is rare in humans with malaria. In humans, SMA is typically associated with parasite burdens that are considerably lower than those required for the marked, direct destruction of RBCs[95], with ~12 uninfected RBCs lost for every PRBC[95,96]. Because of the limitations of hyperparasitaemic experimental infections, there has been increased interest in *Aotus* spp. monkeys either immunized with experimental vaccines consisting of antigens from blood-stage parasites or rendered semi-immune by previous exposure to infection with *P. falciparum*, as these animals develop marked SMA despite having very low parasite burdens[97,98]. Before the advent of antibiotics, the high fever induced by malaria was used to treat neurosyphilis; the low parasite burdens, and the kinetics and magnitude of SMA, in semi-immune simian models mirror the anaemia that developed in the numerous untreated humans who had malaria that was induced for the management of neurosyphilis[95,99,100]. Therefore, because direct destruction of RBCs by parasitization seems to be a relatively

Box 1 | Experimental models of malaria

The study of malaria is aided by the availability of a wide range of experimental models. Various species of the *Plasmodium* genus — such as *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium vinckei* — naturally infect rodents. Adapted to infect laboratory rats and inbred mouse strains, they have proven enormously useful in the development of drugs and vaccines, and in basic studies of pathogenesis, immunology, and the genetics of susceptibility or resistance to infection and disease. Other models use parasites that cause malaria in simians — such as *Plasmodium knowlesi* and *Plasmodium cynomolgi* — or parasites of humans that are adapted to simian hosts (such as *Aotus* spp. monkeys). Although no two host–parasite combinations are identical in all features of the relationship, some models are better than others at recapitulating the main features of pathology or the immune responses that occur in malaria in humans. There is great diversity of responses and disease outcomes in human populations, so experimental models that use inbred hosts might reflect only a section of the natural spectrum of disease in humans: varying both host and parasite genetics uncovers a diversified disease spectrum in these models.

Malaria in mice

Early during infection, *P. berghei* strain ANKA is a good model of cerebral malaria (CM), and processes identified using this model have been subsequently validated in humans. Inbred mouse strains differ markedly in their susceptibility, showing the importance of host genetic variation in immunopathogenesis[133]. Similarly, different strains of *P. berghei* (K173 versus ANKA) differ in some aspects of pathogenesis, indicating the influence of parasite genetic variation in induced pathology.

Infection with *P. yoelii* strain 17XL (a lethal strain) induces CM that is associated with the sequestration of parasitized red blood cells, and it has been used together with *P. yoelii* strain 17XNL (a non-lethal strain) to study experimental vaccine-induced immune responses.

P. chabaudi chabaudi strain AS causes a non-lethal infection in resistant mouse strains and a lethal infection in susceptible mouse strains. Lethality, however, results from haemolysis that is secondary to hyperparasitaemia, which might not be relevant to the human disease processes. This *Plasmodium* strain has been used to study experimental vaccines and immunological processes that control hyperparasitaemia. Infections with *P. chabaudi adami* are self-resolving, non-pathogenic and non-lethal.

P. vinckei vinckei causes an aggressive, overwhelming hyperparasitaemia.

minor contributory mechanism[101], SMA is thought to arise mainly from two processes: increased destruction of non-parasitized RBCs, and decreased production of RBCs (also known as erythropoietic suppression).

Destruction of uninfected RBCs. In SMA in humans, accelerated RBC turnover is proposed to result from changes to the surface or structure of uninfected RBCs that target them for destruction[102]. The following have been detected at relatively high frequencies in individuals with SMA[101]: oxidation, phosphatidylserine externalization and reduced deformability of RBCs; complement binding by RBCs; complement regulatory deficiencies; and autoantibodies, immune complexes and IgG specific for non-specifically adsorbed parasite antigen at the surface of RBCs. These mechanisms are proposed to target RBCs for destruction by intravascular haemolysis or for clearance mediated by the RETICULOENDOTHELIAL SYSTEM (RES). However, human SMA is not associated with the typical signs of intravascular haemolysis, such as secretion of haemoglobin in the urine. By contrast, homeostatic RES clearance of RBCs is mainly mediated by macrophages in the splenic red pulp, and recruitment to the spleen or activation of these macrophages might accelerate the process[103,104]. SMA is associated with high serum levels of neopterin, a marker of macrophage activation, the expression of which is induced by IFN-γ in particular[103]. Phagocytosis of uninfected RBCs has been documented during human infections[105,106], and upregulation of RES activity is implicated in malarial thrombocytopaenia[107]. Anaemia often persists after clearance of parasitaemia, and haemoglobin normalization shows delayed kinetics compared with conditions that have similar blood loss, such as trauma[96]. It is possible that clearance by the RES that involves high RBC turnover extends beyond the period of parasitaemia. In untreated malaria used to control neurosyphilis, the rapid onset of severe anaemia was temporally linked to the appearance of a distended spleen, as was subsequently observed in children with malarial anaemia[99]. These observations are consistent with hyperactivation of the RES and with the magnitude of RBC destruction during SMA. This has led to the proposal that SMA has an inflammatory aetiology[103,104]. Counter-regulatory T_H2 cytokines such as IL-4 (REF. 103) or IL-10 (REFS 108,109) might therefore protect against disease. So, SMA might be regulated by diverse factors that control hypersplenism and splenic macrophage recruitment and activation: for example, host $CD4^+$ T cells, cytokines and chemokines (such as CCL2, CCL3 and CCL4 (also known as MIP1β)) and parasite products (such as haemozoin and GPI). SMA might therefore be precipitated by the rise of parasitaemia above a crucial threshold, as has been observed for experimental infections of simians[97,98] and humans[95,99,100].

Nevertheless, it might be the immune response of the host to this parasite biomass that is the main cause of anaemia, rather than simply the direct destruction of RBCs by parasitization. In broad terms of immunoregulation, SMA might therefore have similarities to other malaria syndromes. Adoptive transfer of parasite-specific, $CD4^+$ T_H1-cell lines promotes anaemia after infection with *P. berghei*[110], showing that antigen-specific T cells can contribute to the severity of disease. A plausible mechanism lies in the ability of T cells to upregulate macrophage-mediated RBC clearance. Because SMA is marked in *Aotus* spp. monkeys that have been immunized with *P. falciparum* antigens[97,98], blood-stage vaccines might not necessarily protect against this condition, although they might suppress parasitaemia. Whether T-cell priming by these experimental vaccines promotes the development of SMA is not clear. Such preclinical models might prove useful for further assessing the efficacy of vaccines in the prevention or promotion of this disease state.

Decreased production of RBCs. Normal homeostasis of RBC numbers is maintained by balancing destruction of old RBCs by the RES with production of new RBCs through erythropoiesis. Under the influence of factors such as erythropoietin, haematopoietic stem cells in the bone marrow or spleen multiply and differentiate to produce the youngest fully functional RBCs, which

RETICULOENDOTHELIAL SYSTEM
The general phagocytic system of the host. It is responsible for removal and destruction of foreign material and senescent or dead host cells, such as red blood cells.

are known as reticulocytes and are the earliest cell in this pathway to be released into circulation. The number of reticulocytes in the blood directly reflects recent erythropoietic activity. Reticulocyte levels are often decreased during acute infection with *P. falciparum*, indicating that another mechanism might contribute to SMA — erythropoietic suppression[111]. Animal models seem to accurately recapitulate this phenomenon. Infection with *Plasmodium chabaudi* or *P. berghei* results in decreased proliferation, differentiation and maturation of erythroid precursors[112,113], and reticulocyte levels are decreased early in infection, with DNA-microarray analysis of splenocytes and bone-marrow cells uncovering reduced transcription of at least 25 erythroid-specific loci[61]. It has been widely proposed that the TNF and IFN-γ cytokine cascade that is associated with the immediate early acute phase of infection mediates erythropoietic suppression in mice and humans, by decreasing the responsiveness of erythroid precursors to erythropoietin[114,115], but this remains unproven. Anaemia in response to acute infection with *P. berghei* strain ANKA is controlled, in part, by genes encoded by the polymorphic NKC loci that are expressed by NK and NKT cells, which control cytokine levels and immune-cell differentiation[37]. Crude *P. berghei* strain ANKA and *P. chabaudi* parasite lysates also induce erythropoietic suppression *in vivo*[116], reflecting the bioactivity of parasite products (possibly haemozoin or GPI), which either directly affect erythroid precursors or indirectly affect them by influencing macrophages (to secrete TNF[3]) or NKT cells[33].

After loss of RBCs in conditions such as trauma, anaemia is normally compensated for by physiological erythropoiesis. However, SMA could be exacerbated if erythropoietic suppression were to prevent adequate reticulocyte compensation during continuing clearance of RBCs. Further investigation of the multiple host and parasite factors that might influence these two contributory mechanisms of SMA *in vivo* is required. Specifically, the impact of vaccination on SMA-related end-points should be examined in more detail in experimental models.

Clinical immunity to malaria

Epidemiological studies show that, after the initial period in which children are susceptible to severe malaria, protective immunity that is acquired to malaria develops in three sequential phases: first, immunity to life-threatening disease; second, immunity to symptomatic infection; and only then, third, partial immunity to parasitization. In 1899, Robert Koch observed that immunity to disease precedes the ability to control parasite densities, as others subsequently observed[117–119], proposing that it reflects the primary acquisition of antitoxic immunity. The data from the treatment of neurosyphilis with malaria shows evidence for antitoxic immunity[120]. At the population level, immunity to severe malaria seems to be acquired after only one or two infections[121], although many children with severe disease have a previous history of multiple mild bouts of malaria.

Several studies report associations between levels of antibodies that are specific for various parasite antigens and reduced risk of infection[122], but this is not clearly established for disease states such as CM or SMA. So, there is no single correlate of clinical immunity, and those described do not account for the overall variation in susceptibility in a population[123]. However, antibodies specific for the parasite glycolipid GPI have been found to be negatively associated with the risk of developing SMA[5] or CM[124] and with acute febrile episodes[125], although a cross-sectional study found no association with tolerance for parasitaemia[126], which might reflect the developmentally compromised acquisition of carbohydrate-specific antibodies in infants. Although clinical immunity might result from adaptive immune responses to GPI, other explanations include the acquisition of physiological non-responsiveness to malaria toxins (which is analogous to tachyphylaxis, the process of downregulation of lipopolysaccharide-responsive signalling pathways following exposure to the agonist). However, this mechanism is unlikely to operate over long time-scales, whereas clinical immunity seems to be relatively robust. A further possibility is that disease susceptibility or resistance is regulated to a considerable extent by the T_H1/T_H2-cytokine profile of the NK- and NKT-cell arm of the immune system (which is intermediate between the innate and adaptive immune systems)[32,33,35–37] or of conventional $CD4^+$ T cells and that clinical immunity is associated with a switch away from the default, T_H1-cell-biased responses to T_H2-cell-biased responses, which prevents severe disease but controls parasite densities only after an appropriately diverse antibody repertoire is generated. Establishing whether clinical immunity results from adaptive immune responses to bioactive parasite products, physiological desensitization to malaria toxins, regulation of the balance of T_H1 and T_H2 cytokines, or a combination of mechanisms is an important issue for future research because such considerations should inform vaccine development.

Implications for vaccines

Vaccines against malaria should aim to reduce morbidity and mortality. Traditional approaches seek to achieve this objective by reducing parasite burdens. In support of this, a recent clinical trial shows that reducing the infective inoculum by administration of a sporozoite-specific vaccine reduces the rates of disease[127]. Nonetheless, reducing the replication of blood-stage parasites, although likely to confer protection, will not necessarily reduce morbidity or mortality, because host immune responses, which can be non-linear with respect to parasite densities, are important determinants of these events. Despite the clinical objective of vaccination, there has been no systematic attempt to assess the impact of experimental vaccines in preclinical models that have appropriate disease end-points. Preclinical models that have been used so far include infection of naive mice with *P. chabaudi* and infection of naive *Aotus* spp. monkeys with *P. falciparum*, and these infections result in high rates of parasite

replication but not in clinically relevant syndromes. As mentioned earlier, SMA is marked in *Aotus* spp. monkeys that have been immunized with *P. falciparum* antigens[97,98], so blood-stage vaccines might not necessarily protect against this condition, although they might suppress parasitaemia. Whether experimental vaccines can induce immune responses that promote SMA is not clear.

The use of vaccines against the disease aims to reduce morbidity and mortality directly, by immunizing individuals with parasite products that contribute to host pathology[128]. For example, vaccines that are designed to prevent malaria during pregnancy target the domains of *P. falciparum* EMP1 that bind placental receptors[129] (that is, CSA and hyaluronic acid). Other organ-specific disease processes could be targeted if a restricted set of *P. falciparum* EMP1 molecules were found to be important[129], the inherent diversity of these targets being a potential barrier to these strategies. Chemical synthesis of the glycan group of *Plasmodium* spp. GPI, which is highly conserved and non-toxic, has recently provided a means to test the hypothesis that this molecule is causally involved in the pathogenesis of *P. berghei* infection[130]. Vaccination with the GPI glycan protects against blood acidosis, pulmonary oedema, vascular occlusion by macrophages, and cerebral fatalities in a rodent model of severe malaria induced by *P. berghei* infection[130]. These findings show the efficacy of a prototype antitoxic vaccine, and they prove that GPI is an essential parasite product in the pathogenesis of systemic disease and in the lethal cerebral syndrome in this model.

It has been argued that antitoxic vaccines might exacerbate disease by inhibiting the acute-phase responses that limit parasite replication[131]. Indeed, it has been proposed that the function of malaria toxins is to elicit host responses that limit infection densities by killing parasites[131]. However, 'suicide' explanations of biological function in unicellular organisms are not easily reconciled with Darwinian logic, it being difficult to envisage why parasites would produce a molecule to cause their own demise. Furthermore, strong acute-phase immune responses are seen in association with high parasite densities in sick children, and marked inflammatory cascades do not necessarily reduce parasite burdens in humans or in experimental models. Clearly, this is an important area for further investigation. Nonetheless, these speculations help to highlight that the impact of the current blood-stage vaccines on malaria pathogenesis remains to be determined, even in preclinical models.

Conclusions

Because they are blood-borne, malaria-causing parasites have access to multiple organs, including the bone marrow, spleen, brain, lungs and placenta. The binding of *P. falciparum* EMP1 to diverse endothelial-cell-expressed receptors concentrates parasites in certain sites. Parasite toxins, such as GPI and haemozoin, induce acute-phase immune responses, with local activation of monocytes and the vascular endothelium. GPI also contributes to early IFN-γ and counter-regulatory IL-4 production, by functioning as a ligand for the CD1d-restricted NKT-cell arm of the immune system. The overall balance of T_H1 and T_H2 cytokines at this stage of the cascade is determined to a considerable extent by the polymorphic loci in the NKC. Chemokine cascades recruit intravascular macrophages and inflammatory cells to diverse target organs, with subsequent deposition of platelets and fibrin. Various lymphoid-cell lineages might further contribute to disease, through the production of pro-inflammatory cytokines, such as IFN-γ. Counter-regulatory T_H2-cell responses might downregulate disease and promote parasite clearance through antibody formation. Much severe pathology in malaria therefore has an immunological basis, although this remains poorly investigated for certain key processes such as metabolic acidosis, which is the strongest prognostic indicator of malarial fatality[88] and one of the least understood aspects of pathogenesis. The importance of immune processes in malaria pathogenesis in humans is further exemplified by clear associations of genetic polymorphisms in immune loci — such as those encoding MBL, CD36, CD40 ligand, TNF, IFN-γ, IL-4 and the p40 subunit of IL-12 — with altered risk of disease[132]. Credible model systems allowing hypothesis testing by experimentation show that multiple convergent factors are required, and few components are sufficient, for disease progression, indicating that there are multiple potential points of intervention in humans. Because T cells contribute to these processes, vaccination strategies should avoid exacerbating disease, by the induction of appropriate counter-regulatory mechanisms or by the neutralization of pathogenic parasite products.

1. Baird, J. K., Masbar, S., Basri, H., Tirtokusumo, S. & Hoffman, S. L. Age-dependent susceptibility to severe disease caused by *Plasmodium falciparum*. *J. Infect. Dis.* **178**, 592–595 (1998).
2. Engwerda, C. R., Beattie, L. & Amante, F. H. The importance of the spleen in malaria. *Trends Parasitol.* **21**, 75–80 (2005).
3. Schofield, L. & Hackett, F. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. *J. Exp. Med.* **177**, 145–153 (1993).
4. Tachado, S. D. *et al.* Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma* and *Leishmania*: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. *Proc. Natl Acad. Sci. USA* **94**, 4022–4027 (1997).
5. Naik, R. S. *et al.* Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *J. Exp. Med.* **192**, 1563–1576 (2000).
6. Carlson, J. *et al.* Disruption of *Plasmodium-falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* **46**, 595–602 (1992).
7. Tachado, S. D. *et al.* Glycosylphosphatidylinositol toxin of *Plasmodium* induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. *J. Immunol.* **156**, 1897–1907 (1996).
8. Schofield, L. *et al.* Glycosylphosphatidylinositol toxin of *Plasmodium* upregulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J. Immunol.* **156**, 1886–1896 (1996).
9. Tachado, S. & Schofield, L. Glycosylphosphatidylinositol of *Trypanosoma brucei* regulates TNF-α and IL-1 gene expression in macrophages by a tyrosine kinase dependent signal transduction pathway. *Biochem. Biophys. Res. Commun.* **205**, 984–991 (1994).
10. Almeida, I. C. *et al.* Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO J.* **19**, 1476–1485 (2000).

11. Debierre-Grockiego, F. *et al.* Roles of glycosytphosphatidyl-inositols of *Toxoplasma gondii*. Induction of tumor necrosis factor-α production in macrophages. *J. Biol. Chem.* **278**, 32987–32993 (2003).
12. Behr, C. *et al. Plasmodium falciparum* stimuli for human γδ T cells are related to phosphorylated antigens of mycobacteria. *Infect. Immun.* **64**, 2892–2896 (1996).
13. Coban, C. *et al.* Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* **201**, 19–25 (2005).
14. Skorokhod, O. A., Alessio, M., Mordmuller, B., Arese, P. & Schwarzer, E. Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-γ-mediated effect. *J. Immunol.* **173**, 4066–4074 (2004).
15. Sherry, B. A. *et al.* Malaria-specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1α, and MIP-1β) *in vitro*, and altered thermoregulation *in vivo*. *J. Inflamm.* **45**, 85–96 (1995).
16. Deshpande, P. & Shastry, P. Modulation of cytokine profiles by malaria pigment — hemozoin: role of IL-10 in suppression of proliferative responses of mitogen stimulated human PBMC. *Cytokine* **28**, 205–213 (2004).
17. Schwarzer, E. *et al.* Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J. Exp. Med.* **176**, 1033–1041 (1992).
18. Morakote, N. & Justus, D. E. Immunosuppression in malaria: effect of hemozoin produced by *Plasmodium berghei* and *Plasmodium falciparum*. *Int. Arch. Allergy Appl. Immunol.* **86**, 28–34 (1988).
19. Scorza, T., Magez, S., Brys, L. & De Baetselier, P. Hemozoin is a key factor in the induction of malaria-associated immunosuppression. *Parasite Immunol.* **21**, 545–554 (1999).
20. Schwarzer, E., Kuhn, H., Valente, E. & Arese, P. Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions. *Blood* **101**, 722–728 (2003).
21. Giribaldi, G. *et al.* Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia. *Haematologica* **89**, 492–493 (2004).
22. Jaramillo, M. *et al.* Hemozoin-inducible proinflammatory events *in vivo*: potential role in malaria infection. *J. Immunol.* **172**, 3101–3110 (2004).
23. Urban, B. *et al. Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* **400**, 73–77 (1999).
24. Urban, B. C., Willcox, N. & Roberts, D. J. A role for CD36 in the regulation of dendritic cell function. *Proc. Natl Acad. Sci. USA* **98**, 8750–8755 (2001).
25. Campos, M. A. *et al.* Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J. Immunol.* **167**, 416–423 (2001).
26. Krishnegowda, G. *et al.* Induction of proinflammatory responses in macrophages by the glycosylphosphatidyl-inositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J. Biol. Chem.* **280**, 8606–8616 (2005).
27. Campos, M. A. *et al.* Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. *J. Immunol.* **172**, 1711–1718 (2004).
28. Adachi, K. *et al. Plasmodium berghei* infection in mice induces liver injury by an IL-12- and Toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J. Immunol.* **167**, 5928–5934 (2001).
29. Kreig, A. A role for Toll in autoimmunity. *Nature Immunol.* **3**, 423–424 (2002).
30. Klabunde, J. *et al.* Recognition of *Plasmodium falciparum* proteins by mannan-binding lectin, a component of the human innate immune system. *Parasitol. Res.* **88**, 113–117 (2002).
31. Luty, A. J., Kun, J. F. & Kremsner, P. G. Mannose-binding lectin plasma levels and gene polymorphisms in *Plasmodium falciparum* malaria. *J. Infect. Dis.* **178**, 1221–1224 (1998).
32. Hansen, D. S. & Schofield, L. Regulation of immunity and pathogenesis in infectious diseases by CD1d-restricted NKT cells. *Int. J. Parasitol.* **34**, 15–25 (2004).
33. Schofield, L. *et al.* CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science* **283**, 225–229 (1999).
34. Fischer, K. *et al.* Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc. Natl Acad. Sci. USA* **101**, 10685–10690 (2004).
35. Hansen, D. S., Siomos, M. A., Buckingham, L., Scalzo, A. A. & Schofield, L. Regulation of murine cerebral malaria pathogenesis by CD1d-restricted NKT cells and the natural killer complex. *Immunity* **18**, 391–402 (2003).
This study shows that NKT cells and loci of the NKC determine T_H1/T_H2-cytokine profiles and therefore susceptibility and resistance to severe malaria in mice.
36. Hansen, D. S. *et al.* CD1d-restricted NKT cells contribute to malarial splenomegaly and enhance parasite-specific antibody responses. *Eur. J. Immunol.* **33**, 2588–2598 (2003).
37. Hansen, D. S. *et al.* The natural killer complex regulates severe malarial pathogenesis and influences acquired immune responses to *Plasmodium berghei* ANKA. *Infect. Immun.* **73**, 2288–2297 (2005).
38. de Kossodo, S. & Grau, G. E. Profiles of cytokine production in relation with susceptibility to cerebral malaria. *J. Immunol.* **151**, 4811–4820 (1993).
39. Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P. & van der Heyde, H. C. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J. Immunol.* **157**, 1620–1624 (1996).
40. Orago, A. & Facer, C. A. Cytotoxicity of human natural killer (NK) cell subsets for *Plasmodium falciparum* erythrocytic schizonts: stimulation by cytokines and inhibition by neomycin. *Clin. Exp. Immunol.* **86**, 22–29 (1991).
41. Theander, T. G., Pedersen, B. K., Bygbjerg, I. C., Jepsen, S. & Larsen, P. B. Enhancement of human natural cytotoxicity by *Plasmodium falciparum* antigen activated lymphocytes. *Acta Trop.* **44**, 415–422 (1987).
42. Artavanis-Tsakonas, K. *et al.* Activation of a subset of human NK cells upon contact with *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* **171**, 5396–5405 (2003).
43. Marchiafava, E. & Bignami, E. in *Twentieth Century Practice of Medicine. An International Encyclopedia of Modern Medical Science* (ed. Stedman, T. L.) 227–252 (Wood & Co., New York, 1900).
44. Coltel, N., Combes, V., Hunt, N. H. & Grau, G. E. Cerebral malaria — a neurovascular pathology with many riddles still to be solved. *Curr. Neurovasc. Res.* **1**, 91–110 (2004).
45. White, N. J. Malaria physiopathology. *Clin. Trop. Med. Commun. Dis.* **1**, 55–90 (1986).
46. Turner, G. Cerebral malaria. *Brain Pathol.* **7**, 569–582 (1997).
47. Molyneux, M. E. Cerebral malaria in children — clinical implications of cytoadherence. *Am. J. Trop. Med. Hyg.* **43**, 38–41 (1990).
48. Grau, G. E. & de Kossodo, S. Cerebral malaria: mediators, mechanical obstruction or more? *Parasitol. Today* **10**, 408–409 (1994).
49. Berendt, A. R. *et al.* Molecular mechanisms of sequestration in malaria. *Parasitology* **108**, S19–S28 (1994).
50. Hunt, N. H. & Grau, G. E. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol.* **24**, 491–499 (2003).
51. Grau, G. E. *et al.* Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J. Infect. Dis.* **187**, 461–466 (2003).
This study indicates that, in CM in humans, similar to the mouse model of CM, there is a close association between the neurological syndrome and the intravascular accumulation of platelets.
52. Rest, J. R. Pathogenesis of cerebral malaria in golden hamsters and inbred mice. *Contrib. Microbiol. Immunol.* **7**, 139–146 (1983).
53. Curfs, J. H. *et al.* Tumour necrosis factor-α and macrophages in *Plasmodium berghei*-induced cerebral malaria. *Parasitology* **107**, 125–134 (1993).
54. Ma, N., Hunt, N. H., Madigan, M. C. & Chan-Ling, T. Correlation between enhanced vascular permeability, up-regulation of cellular adhesion molecules and monocyte adhesion to the endothelium in the retina during the development of fatal murine cerebral malaria. *Am. J. Pathol.* **149**, 1745–1762 (1996).
This paper reports the morphological and functional characterization of the microvascular changes that are associated with experimental CM, using the elegant retinal whole-mount model.
55. Elamin, A. M. Cerebral malaria in adult Zambian Africans. *East Afr. Med. J.* **58**, 124–129 (1981).
56. Pongponratn, E., Riganti, M., Harinasuta, T. & Bunnag, D. Electron microscopy of the human brain in cerebral malaria. *Southeast Asian J. Trop. Med. Public Health* **16**, 219–227 (1985).
57. Porta, J. *et al.* Immunopathological changes in human cerebral malaria. *Clin. Neuropathol.* **12**, 142–146 (1993).
58. Patnaik, J. K. *et al.* Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. *Am. J. Trop. Med. Hyg.* **51**, 642–647 (1994).
59. Mackenzie, C. D. *et al.* Intravascular leukocytes in the brain in Malawian children with fatal malaria. *Am. J. Trop. Med. Hyg.* **61**, 476 (1999).
60. Taylor, T. E. *et al.* Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nature Med.* **10**, 143–145 (2004).
This study highlights the importance of histopathological analyses in our understanding of the neurological syndrome in CM and indicates new approaches to the classification of CM.
61. Sexton, A. C. *et al.* Transcriptional profiling reveals suppressed erythropoiesis, up-regulated glycolysis, and interferon-associated responses in murine malaria. *J. Infect. Dis.* **189**, 1245–1256 (2004).
62. Hanum, P. S., Hayano, M. & Kojima, S. Cytokine and chemokine responses in a cerebral malaria-susceptible or -resistant strain of mice to *Plasmodium berghei* ANKA infection: early chemokine expression in the brain. *Int. Immunol.* **15**, 633–640 (2003).
63. Chen, L., Zhang, Z. & Sendo, F. Neutrophils play a critical role in the pathogenesis of experimental cerebral malaria. *Clin. Exp. Immunol.* **120**, 125–133 (2000).
64. Mohammed, A. O. *et al.* Human neutrophil lipocalin: a specific marker for neutrophil activation in severe *Plasmodium falciparum* malaria. *Acta Trop.* **87**, 279–285 (2003).
65. Wassmer, S. C., Combes, V. & Grau, G. E. Pathophysiology of cerebral malaria: role of host cells in the modulation of cytoadhesion. *Ann. NY Acad. Sci.* **992**, 30–38 (2003).
66. Hawrylowicz, C. M., Howells, G. L. & Feldmann, M. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J. Exp. Med.* **174**, 785–790 (1991).
67. Barry, O. P., Pratico, D., Lawson, J. A. & FitzGerald, G. A. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J. Clin. Invest.* **99**, 2118–2127 (1997).
68. Barry, O. P., Pratico, D., Savani, R. C. & FitzGerald, G. A. Modulation of monocyte–endothelial cell interactions by platelet microparticles. *J. Clin. Invest.* **102**, 136–144 (1998).
69. Combes, V. *et al. ABCA1* gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology. *Am. J. Pathol.* **166**, 295–302 (2005).
70. Combes, V. *et al.* Circulating endothelial microparticles in Malawian children with severe falciparum malaria complicated with coma. *JAMA* **291**, 2542–2544 (2004).
71. Kuijper, P. H. *et al.* Platelet associated fibrinogen and ICAM-2 induce firm adhesion of neutrophils under flow conditions. *Thromb. Haemost.* **80**, 443–448 (1998).
72. Kuijper, P. H. *et al.* Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. *Blood* **87**, 3271–3281 (1996).
73. van der Heyde, H. C., Gramglia, I., Sun, G. & Woods, C. Platelet depletion by anti-CD41 (α_{IIb}) mAb injection early but not late in the course of disease protects against *Plasmodium berghei* pathogenesis by altering the levels of pathogenic cytokines. *Blood* **105**, 1956–1963 (2005).
74. Sun, G. *et al.* Inhibition of platelet adherence to brain microvasculature protects against severe *Plasmodium berghei* malaria. *Infect. Immun.* **71**, 6553–6561 (2003).
75. Grau, G. E. *et al.* L3T4$^+$ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. *J. Immunol.* **137**, 2348–2354 (1986).
76. Yanez, D. M., Batchelder, J., van der Heyde, H. C., Manning, D. D. & Weidanz, W. P. γδ T-cell function in pathogenesis of cerebral malaria in mice infected with *Plasmodium berghei* ANKA. *Infect. Immun.* **67**, 446–448 (1999).
77. Grau, G. E. *et al.* Monoclonal antibody against interferon-γ can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. *Proc. Natl Acad. Sci. USA* **86**, 5572–5574 (1989).
78. Amani, V. *et al.* Involvement of IFN-γ receptor-mediated signaling in pathology and anti-malarial immunity induced by *Plasmodium berghei* infection. *Eur. J. Immunol.* **30**, 1646–1655 (2000).
79. Jacobs, T., Graefe, S. E., Niknafs, S., Gaworski, I. & Fleischer, B. Murine malaria is exacerbated by CTLA-4 blockade. *J. Immunol.* **169**, 2323–2329 (2002).
80. Hermsen, C. C., Crommert, J. V. D., Fredrix, H., Sauerwein, R. W. & Eling, W. M. C. Circulating tumour necrosis factor α is not involved in the development of cerebral malaria in *Plasmodium berghei*-infected C57Bl mice. *Parasite Immunol.* **19**, 571–577 (1997).

81. Belnoue, E. *et al.* On the pathogenic role of brain-sequestered αβ CD8$^+$ T cells in experimental cerebral malaria. *J. Immunol.* **169**, 6369–6375 (2002).
82. Potter, S., Chaudhri, G., Hansen, A. & Hunt, N. H. Fas and perforin contribute to the pathogenesis of murine cerebral malaria. *Redox Rep.* **4**, 333–335 (1999).
83. Nitcheu, J. *et al.* Perforin-dependent brain-infiltrating cytotoxic CD8$^+$ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J. Immunol.* **170**, 2221–2228 (2003).
84. Boubou, M. I. *et al.* T cell response in malaria pathogenesis: selective increase in T cells carrying the TCR Vβ8 during experimental cerebral malaria. *Int. Immunol.* **11**, 1553–1562 (1999).
85. Belnoue, E. *et al.* Chemokine receptor CCR2 is not essential for the development of experimental cerebral malaria. *Infect. Immun.* **71**, 3648–3651 (2003).
86. Lou, J., Dayer, J. M., Grau, G. E. & Burger, D. Direct cell/cell contact with stimulated T lymphocytes induces the expression of cell adhesion molecules and cytokines by human brain microvascular endothelial cells. *Eur. J. Immunol.* **26**, 3107–3113 (1996).
87. Chang, W. L. *et al.* CD8$^+$-T-cell depletion ameliorates circulatory shock in *Plasmodium berghei*-infected mice. *Infect. Immun.* **69**, 7341–7348 (2001).
88. Marsh, K., English, M. & Crawley, J. The pathogenesis of severe malaria in African children. *Ann. Trop. Med. Parasitol.* **90**, 395–402 (1996).
This paper outlines the importance of metabolic acidosis in severe disease.
89. Fried, M. & Duffy, P. E. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**, 1502–1504 (1996).
90. Rowe, J. A. & Kyes, S. A. The role of *Plasmodium falciparum var* genes in malaria in pregnancy. *Mol. Microbiol.* **53**, 1011–1019 (2004).
91. Jerusalem, C. *et al.* Comparative clinical and experimental study on the pathogenesis of cerebral malaria. *Contrib. Microbiol. Immunol.* **7**, 130–138 (1983).
92. Ordi, J. *et al.* Massive chronic intervillositis of the placenta associated with malarial infection. *Am. J. Surg. Pathol.* **22**, 1006–1011 (1998).
93. Rogerson, S. J. *et al.* Placental monocyte infiltrates in response to *Plasmodium falciparum* malaria infection and their association with adverse pregnancy outcomes. *Am. J. Trop. Med. Hyg.* **68**, 115–119 (2003).
94. McElroy, P. D. *et al.* Effect of *Plasmodium falciparum* parasitemia density on hemoglobin concentrations among full-term, normal birth weight children in western Kenya, IV. The Asembo Bay Cohort Project. *Am. J. Trop. Med. Hyg.* **62**, 504–512 (2000).
95. Jakeman, G. N., Saul, A., Hogarth, W. L. & Collins, W. E. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology* **119**, 127–133 (1999).
This is a retrospective analysis of neurosyphilis-management data sets, and it shows that experimentally induced malaria leads to anaemia of low parasite burden, owing to loss of uninfected (non-parasitized) RBCs.
96. Price, R. N. *et al.* Factors contributing to anemia after uncomplicated falciparum malaria. *Am. J. Trop. Med. Hyg.* **65**, 614–622 (2001).
97. Egan, A. F., Fabucci, M. E., Saul, A., Kaslow, D. C. & Miller, L. H. *Aotus* New World monkeys: model for studying malaria-induced anemia. *Blood* **99**, 3863–3866 (2002).
98. Jones, T. R. *et al.* Anemia in parasite- and recombinant protein-immunized *Aotus* monkeys infected with *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **66**, 672–679 (2002).
References 97 and 98 show that severe anaemia develops in the preclinical model in which *Aotus* spp. monkeys are challenged with *P. falciparum* following effective vaccination with blood-stage parasite antigens.
99. Kitchen, S. F. in *Malariology* (ed. Boyd, M. F.) 995–1016 (Saunders, Philadelphia, 1949).
100. Collins, W. E., Jeffery, G. M. & Roberts, J. M. A retrospective examination of anemia during infection of humans with *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* **68**, 410–412 (2003).
101. Ekvall, H. Malaria and anemia. *Curr. Opin. Hematol.* **10**, 108–114 (2003).
102. Looareesuwan, S. *et al.* Erythrocyte survival in severe falciparum malaria. *Acta Trop.* **48**, 372–373 (1991).
103. Biemba, G., Gordeuk, V. R., Thuma, P. E., Mabeza, G. F. & Weiss, G. Prolonged macrophage activation and persistent anaemia in children with complicated malaria. *Trop. Med. Int. Health* **3**, 60–65 (1998).
104. Biemba, G., Gordeuk, V. R., Thuma, P. & Weiss, G. Markers of inflammation in children with severe malarial anaemia. *Trop. Med. Int. Health* **5**, 256–262 (2000).
105. Abdalla, S. H. Peripheral blood and bone marrow leucocytes in Gambian children with malaria: numerical changes and evaluation of phagocytosis. *Ann. Trop. Paediatr.* **8**, 250–258 (1988).
106. La Raja, M. Erythrophagocytosis by peripheral monocytes in *Plasmodium falciparum* malaria. *Haematologica* **87**, EIM14 (2002).
107. Erhart, L. M. *et al.* Hematologic and clinical indices of malaria in a semi-immune population of western Thailand. *Am. J. Trop. Med. Hyg.* **70**, 8–14 (2004).
108. Kurtzhals, J. A. *et al.* Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet* **351**, 1768–1772 (1998).
109. Othoro, C. *et al.* A low interleukin-10 tumor necrosis factor-α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. *J. Infect. Dis.* **179**, 279–282 (1999).
110. Hirunpetcharat, C., Finkelman, F., Clark, I. A. & Good, M. F. Malaria parasite-specific T_H1-like T cells simultaneously reduce parasitemia and promote disease. *Parasite Immunol.* **21**, 319–329 (1999).
111. Kurtzhals, J. A. *et al.* Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falciparum* malaria. *Br. J. Haematol.* **97**, 169–174 (1997).
112. Villeval, J. L., Lew, A. & Metcalf, D. Changes in hemopoietic and regulator levels in mice during fatal or nonfatal malarial infections. I. Erythropoietic populations. *Exp. Parasitol.* **71**, 364–374 (1990).
113. Chang, K. H., Tam, M. & Stevenson, M. M. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. *Blood* **103**, 3727–3735 (2004).
114. Chang, K. H. & Stevenson, M. M. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int. J. Parasitol.* **34**, 1501–1516 (2004).
115. McDevitt, M. A., Xie, J., Gordeuk, V. & Bucala, R. The anemia of malaria infection: role of inflammatory cytokines. *Curr. Hematol. Rep.* **3**, 97–106 (2004).
116. Rudin, W., Quesniaux, V., Favre, N. & Bordmann, G. Malaria toxins from *P. chabaudi chabaudi* AS and *P. berghei* ANKA cause dyserythropoiesis in C57BL/6 mice. *Parasitology* **115**, 467–474 (1997).
117. Christophers, S. R. The mechanism of immunity against malaria in communities living under hyperendemic conditions. *Indian J. Med. Res.* **12**, 273–294 (1924).
118. Sinton, J. A. A summary of our present knowledge of the mechanism of immunity in malaria. *J. Malaria Inst. India* **2**, 71–83 (1939).
119. McGregor, I. A., Giles, H. M., Walters, J. H., Davies, A. H. & Pearson, F. A. Effects of heavy and repeated malarial infections on Gambian infants and children. *Br. Med. J.* **2**, 686–692 (1956).
120. Molineaux, L., Trauble, M., Collins, W. E., Jeffery, G. M. & Dietz, K. Malaria therapy reinoculation data suggest individual variation of an innate immune response and independent acquisition of antiparasitic and antitoxic immunities. *Trans. R. Soc. Trop. Med. Hyg.* **96**, 205–209 (2002).
121. Gupta, S., Snow, R. W., Donnelly, C. A., Marsh, K. & Newbold, C. Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nature Med.* **5**, 340–343 (1999).
References 117–121 elucidate that antitoxic immunity precedes, and is dissociable from, antiparasite immunity and that it protects hosts after only a few infections.
122. Polley, S. D. *et al.* Human antibodies to recombinant protein constructs of *Plasmodium falciparum* apical membrane antigen 1 (AMA1) and their associations with protection from malaria. *Vaccine* **23**, 718–728 (2004).
123. Mackintosh, C. L., Beeson, J. G. & Marsh, K. Clinical features and pathogenesis of severe malaria. *Trends Parasitol.* **20**, 597–603 (2004).
124. Perraut, R. B. *et al.* Differential antibody responses to *Plasmodium falciparum* glycosylphosphatidylinositol anchors in patients with cerebral and mild malaria. *Microbes Infect.* **7**, 682–687 (2005).
125. Hudson Keenihan, S. N. *et al.* Age-dependent impairment of IgG responses to glycosylphosphatidylinositol with equal exposure to *Plasmodium falciparum* among Javanese migrants to Papua, Indonesia. *Am. J. Trop. Med. Hyg.* **69**, 36–41 (2003).
126. Boutlis, C. S. *et al.* Antibodies to *Plasmodium falciparum* glycosylphosphatidylinositols: inverse association with tolerance of parasitemia in Papua New Guinean children and adults. *Infect. Immun.* **70**, 5052–5057 (2002).
127. Alonso, P. L. *et al.* Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. *Lancet* **364**, 1411–1420 (2004).
128. Schofield, L. Antidisease vaccines. *Chem. Immunol.* **80**, 322–342 (2002).
129. Smith, J. D. & Deitsch, K. W. Pregnancy-associated malaria and the prospects for syndrome-specific antimalaria vaccines. *J. Exp. Med.* **200**, 1093–1097 (2004).
130. Schofield, L., Hewitt, M. C., Evans, K., Siomos, M. A. & Seeberger, P. H. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* **418**, 785–789 (2002).
This paper shows a causal role for GPI in the pathogenesis of malaria in rodents, and it provides proof of principle for the development of an antitoxic vaccine against malaria.
131. Kwiatkowski, D. TNF-inducing malaria toxin: a sheep in wolf's clothing? *Ann. Trop. Med. Parasitol.* **87**, 613–616 (1993).
132. Kwiatkowski, D. Genetic susceptibility to malaria getting complex. *Curr. Opin. Genet. Dev.* **10**, 320–324 (2000).
133. Lou, J., Lucas, R. & Grau, G. E. Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. *Clin. Microbiol. Rev.* **14**, 810–820 (2001).

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Competing interests statement

The authors declare no competing financial interests.

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Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes

Krystal J. Evans, Diana S. Hansen, Nico van Rooijen, Lynn A. Buckingham, and Louis Schofield

Severe malarial anemia (SMA) is the most frequent life-threatening complication of malaria and may contribute to the majority of malarial deaths worldwide. To explore the mechanisms of pathogenesis, we developed a novel murine model of SMA in which parasitemias peaked around 1.0% of circulating red blood cells (RBCs) and yet hemoglobin levels fell to 47% to 56% of baseline. The severity of anemia was independent of the level of peak or cumulative parasitemia, but was linked kinetically to the duration of patent infection. In vivo biotinylation analysis of the circulating blood compartment revealed that anemia arose from accelerated RBC turnover. Labeled RBCs were reduced to 1% of circulating cells by 8 days after labeling, indicating that the entire blood compartment had been turned over in approximately one week. The survival rate of freshly transfused RBCs was also markedly reduced in SMA animals, but was not altered when RBCs from SMA donors were transferred into naive recipients, suggesting few functional modifications to target RBCs. Anemia was significantly alleviated by depletion of either phagocytic cells or CD4$^+$ T lymphocytes. This study demonstrates that immunologic mechanisms may contribute to SMA by promoting the accelerated turnover of uninfected RBCs. (Blood. 2006;107: 1192-1199)

Introduction

Plasmodium falciparum malaria infects 10% of the global population, causing 2 million fatalities annually.[1] Severe malarial anemia (SMA) is the most prevalent serious complication of malaria, the commonest life-threatening syndrome, and may be the leading cause of malarial deaths worldwide.[2] Despite high relevance to human welfare, the molecular and cellular basis of SMA remains obscure.

SMA is thought to arise from both decreased red blood cell (RBC) production and increased RBC destruction. Destruction of RBCs can occur as a result of parasite invasion and replication; however, in malaria-endemic areas SMA is consistently observed at relatively low parasite burdens. For example, in Gambian children SMA was associated with a geometric mean of parasite density of 10 470 parasites per μL, around 0.2% of circulating cells.[3] Mathematic modeling of hematologic data from experimental human *P falciparum* infections,[4] as well as analysis of clinical data from endemic areas,[5] has suggested that up to 12 uninfected RBCs (uRBCs) are lost for every infected RBC. Thus it is widely accepted that the direct destruction of RBCs following parasitization cannot account for the degree of anemia observed during malaria infection, suggesting that the destruction of uRBCs is the major cause of hemoglobin (Hb) loss.[6]

Increased destruction of uRBCs is proposed to result from mechanisms such as bystander intravascular hemolysis or accelerated senescence, arising from lipid peroxidation,[7] reduced red cell deformability,[8] modification by surface-bound IgG or complement,[9] up-regulation of host phagocytic function,[10] and adsorption of parasite-derived antigens.[11] Many of these proposed mechanisms are derived from observational studies of human malaria infection in endemic areas. Correlative clinical data often require further experimental hypothesis testing to determine casual processes, particularly within in vivo experimental models. However, the study of RBC destruction in rodent models of malaria is confounded by the development of hyperparasitemia, with levels of infection peaking between 25% to 75% of circulating RBCs,[12] resulting in a predominantly hemolytic anemia. Hyperparasitemia in humans is uncommon in malaria holoendemic areas and is often defined as more than 5% infection of peripheral RBCs (more than 250 000 parasites/μL). Based on these definitions, almost all naive murine malaria infections can be classified as hyperparasitemic, and the associated hemolytic anemia may not be reflective of SMA in human populations.

Therefore, in this study, we sought to investigate the destruction of RBCs in rodent models of SMA that are uncomplicated by excessive parasite burdens. The World Health Organization (WHO) definition of SMA is hemoglobin (Hb) levels less than 50 g/L (5 g/dL) in the presence of parasitemia of at least 10 000/μL (0.2% of peripheral RBCs) and a normocytic blood film.[13] We observed the development of this degree of anemia with low parasite burdens in semi-immune BALB/c mice and in naive Wistar rats infected with *P berghei* ANKA, with kinetic features similar to those observed in experimental malaria infections of semi-immune *Aotus*[14,15] and naive humans.[4,16] Using these models, we demonstrate that SMA arises from the accelerated turnover of uRBCs, and that phagocytic

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cells and CD4+ T lymphocytes are significantly involved in disease pathogenesis. This suggests that SMA may be mediated in part by immunopathologic processes.[17]

Materials and methods

Rodent malaria infections and profiles of SMA

BALB/c mice aged 7 to 8 weeks or 15-week-old Wistar rats were injected intraperitoneally with 10^4 or 10^6 *P berghei* ANKA–infected RBCs, respectively. Parasite and reticulocyte levels were monitored every 2 days by Giemsa-stained thin blood film and are expressed as a percentage of more than 500 RBCs. The day of patency was determined following microscopic examination of 5000 RBCs. Hb was measured by absorbance at 540 nm of 4 μL tail-vein blood suspended in 1 mL Drabkin reagent (Sigma, St Louis, MO) and is expressed as a percentage of baseline levels. Hematocrit (Hct) was determined by diluting 2 μL tail-vein blood in 5 mL PBS and counting by hemocytometer. Hb and Hct were tightly correlated, and Hct was calculated using percentage change in Hb level and a resting Hct of 6×10^9 cells/mL.

Generation of semi-immune mice

Cohorts of infected mice were treated at day 6 after infection with chloroquine (10 mg/kg intraperitoneally) and pyrimethamine (10 mg/kg intraperitoneally) daily for 5 days. Mice were rested for 2 weeks and rechallenged with 10^4 *P berghei* ANKA. During subsequent rounds of infection, mice were monitored and drug-cured prior to parasitemias reaching 5%. Mice underwent 4 to 5 cycles of drug-cured infection before being challenged with 10^4 *P berghei* parasites.

Phenylhydrazine treatment

Mice were injected intravenously with 60 mg/kg phenylhydrazine (PHZ; Sigma), or PBS, on 2 consecutive days. Hb from tail-vein blood was quantified in Drabkin reagent using a 3-point triangulation method (500, 540, and 600 nm).

In vivo biotinylation of RBCs

RBCs of semi-immune mice and naive rats were labeled in vivo by intravenous injection of EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) in PBS at dosages of 0.75 to 1.0 mg per mouse and 3.0 to 4.5 mg per rat. For transfer experiments, RBCs were collected by heparinized cardiac puncture 4 hours after labeling, washed, and resuspended in PBS.

Carboxy-fluorescein succinimidyl ester (CFSE) labeling of RBCs

RBCs from naive mice were harvested by heparinized cardiac puncture and washed 3 times in warm PBS. Cells were incubated with 10 μM CFSE (Molecular Probes, Eugene, OR) for 30 minutes in the dark at 37°C, washed, and resuspended in PBS.

RBC transfers

Labeled RBCs were injected intravenously into naive and semi-immune mice (100-150 μL). The total number of labeled cells transferred was calculated from flow cytometry positive percentages multiplied by Hct. Survival rates of labeled cells are expressed as a percentage of the initial transferred population. Naive mice receiving RBCs from infected donors were maintained on pyrimethamine-treated drinking water (70 mg/L) to prevent the establishment of *P berghei* infection.

Flow cytometry of biotinylated RBCs

Heparinized tail-vein blood (2 μL) was washed with PBS, incubated with strepavidin-conjugated phycoerythrin (PE; Pharmingen, San Diego, CA) at room temperature in the dark for 30 minutes, washed again, resuspended in PBS, and analyzed by flow cytometry.

In vivo depletion of phagocytic cells

Clodronate (gift of Roche Diagnostics, Mannheim, Germany) was encapsulated in liposomes as described earlier.[18] *P berghei*–infected semi-immune mice received 200 μL intravenously of clodronate-liposomes in PBS or PBS alone on day of patency and 150 μL intravenously 4 days after patency.

In vivo depletion of CD4+ T cells

P berghei–infected semi-immune mice received 300 μg rat antimouse CD4 mAb (GK1.5) or an isotype-matched control rat mAb (GL121) in PBS intravenously on day of patency, and 300 μg intraperitoneally on days 2, 4, 6, 8, and 10 after patency. Flow cytometry revealed less than 0.1% splenic CD4+ cells 24 and 48 hours after GK1.5 administration.

Statistical analysis

Data are expressed as the mean plus or minus the standard error about the mean (SEM) and Student unpaired *t* tests were performed. A permutation test devised by Dr Russell Thomson (WEHI Bioinformatics Group)[19] was used to assess differences between Hb profiles over the course of infection.

Results

Semi-immune mouse model of SMA at low parasite burden

Semi-immune BALB/c mice were generated by successive cycles of infection with 10^4 *P berghei* followed by antimalarial drug-cure. When challenged with 10^4 *P berghei*, the kinetics of infection in the semi-immune cohort varied considerably between individuals (Figure 1A) and the day of patency occurred between day 10 to day 22 after infection. Independent of the time-to-patency, parasitemias resolved rapidly, without chemotherapy, within 6 days of patency. The peak values and kinetics experienced by individual animals were averaged (Table 1). In semi-immune mice, the average peak parasitemia was 0.94%, which occurred approximately 2 days after patency. Naive, age-matched BALB/c inoculum controls developed parasite burdens in excess of 60% by day 16 after infection (Figure 1B). At this time, naive mice were severely anemic, with Hb levels at 30% of baseline. Naive animals displayed a high incidence and severity of hematuria, and anemia was attributed to the hemolysis of infected RBCs. In contrast, semi-immune mice experienced severe anemia in response to a self-resolving, low burden of parasitemia (< 1%), with rapid decreases in Hb following the peak in parasitemia (Figure 1A). Minimum Hb levels were seen 4.1 days after patency, 2 days after the peak in parasitemia, representing a loss of 45% of baseline Hb in 96 hours. Hb levels were strongly correlated with Hct ($R^2 = 0.835$), consistent with the normochromic profile of SMA in humans. Reticulocyte levels peaked at 35% of circulating cells at 5.9 days after patency, 2 days after the anemic crisis, and returned to baseline upon the resolution of anemia. The correlation between minimum Hb and maximum parasitemia was very poor ($R^2 = 0.073$, Figure 1C), indicating that the degree of anemia was independent of the level of peak parasitemia. Although the day of patency was highly variable, it did not impact the development of anemia as minimum Hb levels were independent of the time to patency ($R^2 = 0.054$). Following patency, however, the subsequent kinetics of infection, anemia, and compensatory reticulocytosis were very tightly linked (Table 1). The data from individual mice were normalized to the day of

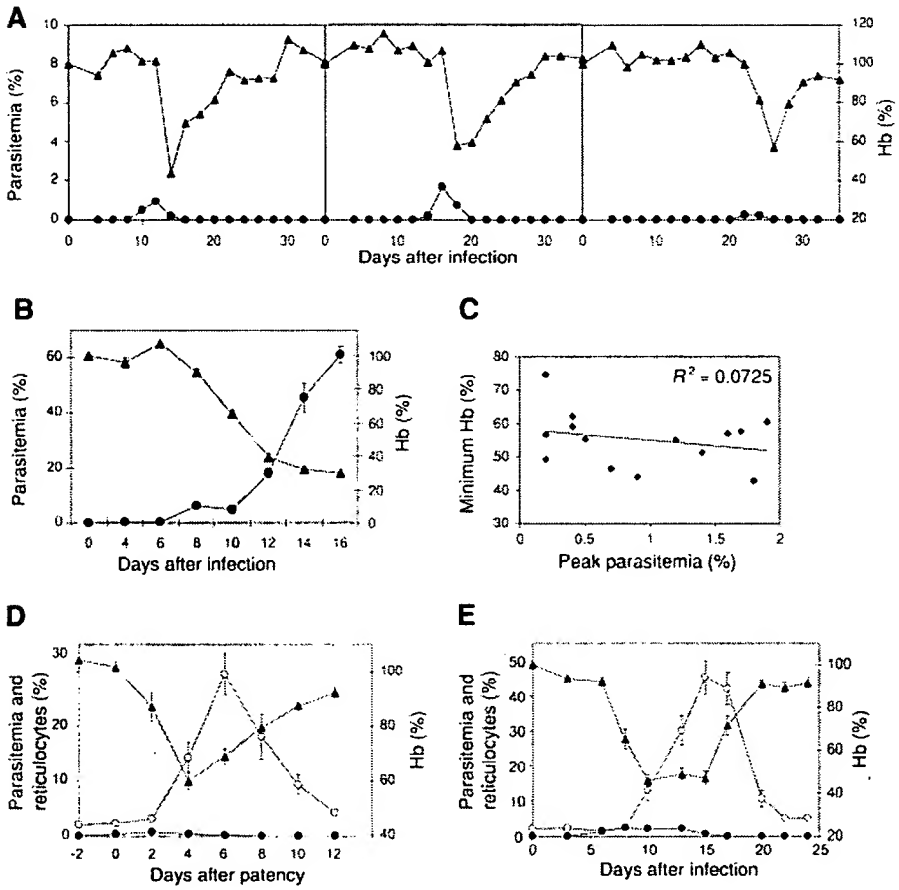

Figure 1. Severe malarial anemia at low parasite burden in rodent malaria infections. (A) Representative data from 3 infected semi-immune BALB/c mice showing parasitemia (●) and Hb levels (▲) following infection with 10^4 *P berghei*. (B) Parasitemia (●) and Hb levels (▲) of naive inoculum control BALB/c mice. (C) Lack of correlation between peak parasitemia and minimum Hb levels in infected semi-immune mice. (D) Parasitemia (●), reticulocyte levels (○), and Hb (▲) of semi-immune mice normalized to the day of patency. (E) Mean parasitemias (●), reticulocyte levels (○), and Hb (▲) in naive adult rats infected with *P berghei*. n = 14 ± SEM for immune mice; n = 6 ± SEM for naive mice and naive rats.

patency (Figure 1D), revealing very low variance in the data set and establishing a novel murine model of SMA at low parasite burden.

Naive rat model of SMA at low parasite burden

We sought to determine whether SMA at low parasite burden could be replicated in naive rodent hosts. Naive Wistar rats aged 15 weeks were challenged with 10^6 *P berghei*, and parasitemia, Hb, and reticulocyte levels were determined over the course of infection (Figure 1E) as well as mean minimum and maximum values (Table 2). The period of patent infection was protracted, beginning on days 6 to 8 after infection, peaking at 3.4% iRBC and resolving by days 15 to 17 after infection. Following the onset of patency, there was a rapid and considerable loss of Hb beginning on days 8 to 10 after infection, with prolonged anemia reaching an average nadir of 39% of baseline on day 13.0 after infection. Sustained reticulocyte production occurred in response to anemia, with a sharp escalation in circulating reticulocytes at day 13, which remained high until day 17. This facilitated an increase in Hb levels and reticulocyte levels returned to basal levels as rats recovered from anemia.

The period and duration of patent infection was linked to the period and duration of anemia in both the semi-immune mouse model and the naive rat model of SMA. Semi-immune mice experienced an average of 4.6 ± 0.5 days of patent parasitemia and 5.1 ± 0.5 days of anemia (< 75% of baseline Hb). In naive rats, patent infections lasted an average of 10.3 ± 0.2 days and the corresponding period of anemia was 9.9 ± 0.2 days. The cumulative parasitemias over the course of infection could not account for the magnitude of anemia experienced, and while the degree of anemia was independent of peak parasitemias (Figure 1C), the duration of patency and duration of anemia were closely associated in both rodent models.

Reticulocyte production during SMA

To assess the responsiveness of the erythropoietic compartment, reticulocyte production during SMA was examined in semi-immune mice. The peak level of circulating reticulocytes correlated with the degree of anemia (Figure 2A). To determine whether the magnitude of the erythropoietic response was physiologically appropriate, reticulocyte production during SMA was compared

Table 1. The average kinetics and magnitude of peak parasitemia, minimum Hb, and peak reticulocyte levels during *P berghei* infection of semi-immune mice

	Peak day after patency	Peak value, %
Parasitemia	1.7 ± 0.40	0.94 ± 0.17
Minimum Hb	4.1 ± 0.25	55.2 ± 2.2
Reticulocytes	5.9 ± 0.39	34.8 ± 3.9

n = 14 ± SEM.

Table 2. Magnitude and kinetics of individual peak parasitemia, minimum Hb, and peak reticulocyte levels during *P berghei* infection of naive rats

	Peak day after patency	Peak value, %
Parasitemia	9.6 ± 0.6	3.4 ± 0.5
Minimum Hb	13.0 ± 0.9	39.0 ± 1.4
Reticulocytes	16.0 ± 0.3	52.0 ± 4.2

n = 6 ± SEM.

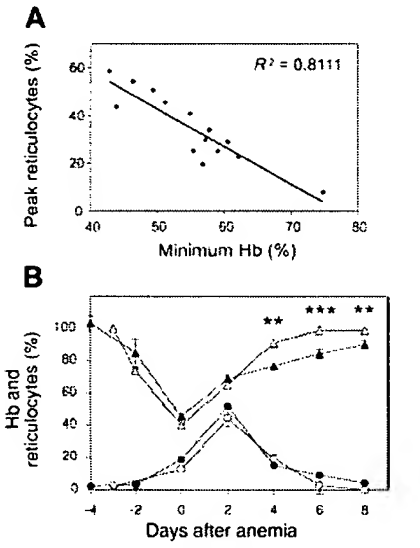

Figure 2. Erythropoietic response to severe malarial anemia in semi-immune mice. (A) Correlation between peak reticulocyte levels and minimum Hb levels during SMA in semi-immune mice (n = 14). (B) Hb (▲ and △) and reticulocyte (● and ○) levels ± SEM in mice with SMA (▲ and ●, n = 4) or nonmalarial PHZ-induced anemia (△ and ○, n = 6). Mice were selected for equivalent levels of anemia and data are normalized to the day of peak anemia (day = 0). $^{**}P < .01$; $^{***}P < .001$.

with that in mice with similar losses of Hb due to nonmalarial causes. The administration of PHZ in vivo results in the hemolysis of RBCs, inducing a dose-dependent anemia with appropriate compensatory erythropoiesis. PHZ-treated mice experienced a reduction of Hb to 40% of baseline levels, and a subset of mice with comparable levels of SMA was selected from a semi-immune cohort and data were normalized to the day of minimum Hb (Figure 2B). Intravascular hemolysis caused pronounced hematuria in PHZ-treated mice, as was observed in naive hyperparasitemic animals. However, this condition was not observed in the semi-immune SMA model despite a similar magnitude of anemia, indicating that intravascular hemolysis was not a major contributory mechanism to SMA in the semi-immune model. The kinetics and magnitude of reticulocyte production were similar in both PHZ-treated and SMA mice, with reticulocyte responses peaking 2 days after minimum Hb levels at 45% and 50%, respectively. Thus, the erythropoietic response to SMA was intact and appropriate for the degree of anemia experienced. Hb levels in PHZ-treated mice returned to baseline 7.0 ± 1.0 days after reaching minimum levels. Despite comparable levels of severe anemia and compensatory reticulocytosis, malaria-infected mice had significantly reduced Hb levels on days 4, 6, and 8 ($P < .01$) (Figure 2B) and took 12 ± 1.3 days to return to baseline (not shown). This reduced capacity to restore Hb suggests ongoing destruction or compromised survival of RBCs during recovery from SMA.

Accelerated RBC turnover during SMA

To determine the survival rates of RBCs in the semi-immune model of SMA, in vivo biotinylation of the circulating blood compartment was undertaken on the day of patency and cell survival was traced by flow cytometry (Figure 3A). Biotinylation of RBCs is widely used in experimental and clinical settings[20,21] and revealed no impact on RBC survival in vivo (Figure 3A, inset). During the course of SMA, there was a 50% reduction in labeled RBCs between day 0 and day 4 after patency, with a concurrent 49% reduction in Hb levels (Figure 3A), demonstrating that the clearance of RBCs from circulation accounts for the severity of anemia. Between days 4 and 8 after patency, Hb levels stabilized and began to rise; however, the level of labeled RBCs continued to decrease, falling to 1% of circulating cells by day 8 after patency. This indicated that the entire initial blood compartment (9×10^9 cells) had been turned over in approximately one week (Figure 3B). Animals were followed for 20 days after patency till baseline Hb levels were restored, and labeled RBCs did not reappear in circulation throughout this time (data not shown). This demonstrates that labeled RBCs did not participate in the restoration of Hb levels and suggests that the RBCs had been destroyed.

The extent and rate of RBC clearance in the naive Wistar rat model of SMA was determined by in vivo biotinylation of circulating RBCs at different time points following *P berghei* infection (Figure 3C). Cells were labeled on day 6, the day of patency; day 10, the day of peak anemia; and day 13, the midpoint of the anemic crisis, and showed similar rates of clearance from circulation (Figure 3C). A 50% reduction in the total number of labeled RBCs in circulation was observed 4.3, 3.2, and 4.0 days from days 6, 10, and 13 after infection, respectively, indicating that accelerated RBC turnover occurred at similar rates throughout the course of infection. Of interest, the level of biotinylated RBCs continued to decrease between days 17 and 24 after infection

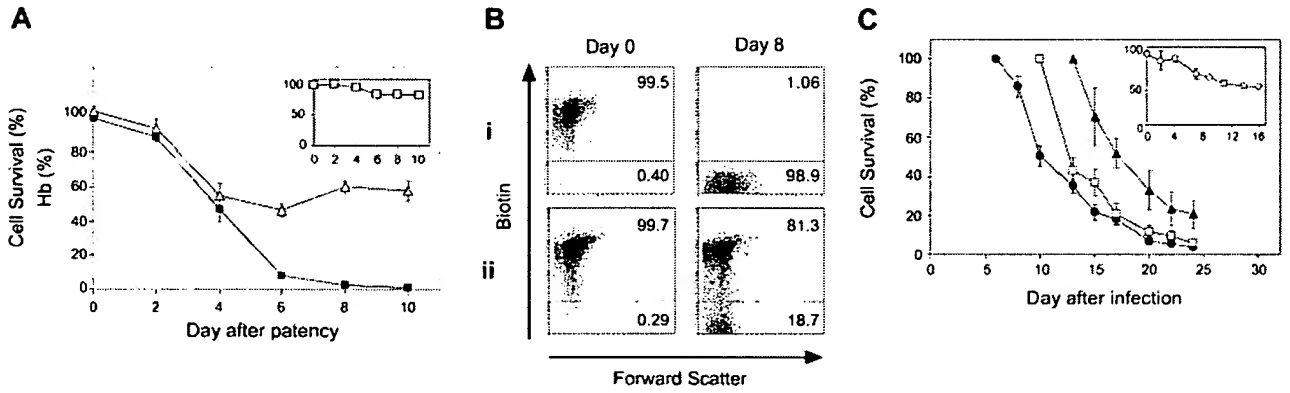

Figure 3. Accelerated RBC turnover during severe malarial anemia. (A) Survival of the circulating RBC population biotinylated on the day of patency in semi-immune mice infected with *P berghei* (■) and Hb levels (△). Basal turnover of biotinylated RBCs in noninfected mice is shown in inset (□). Cell survival was monitored by flow cytometry and is expressed as a percentage change in total number of initial biotinylated RBCs. n = 4 ± SEM. (B) Representative flow cytometry profile of biotinylated RBCs in (i) an infected semi-immune mouse and (ii) naive resting control mouse on days 0 (day of labeling) and 8 after patency. (C) Survival of circulating RBC populations in naive rats, biotinylated on day 6 (●), day 10 (□), and day 13 (▲) after patency. Basal turnover of biotinylated RBCs in uninfected rats is shown in inset (○). Cell survival was monitored by flow cytometry and is expressed as a percentage change in total number of initial biotinylated RBCs. n = 3 ± SEM per group.

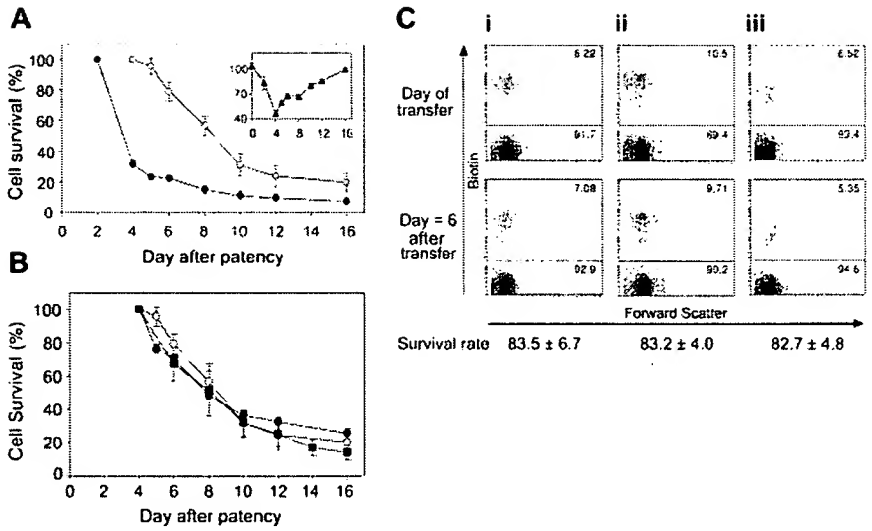

Figure 4. RBC fate is not determined by changes to target cells. (A) Naive RBCs were labeled with CSFE (●) or biotin (○) and adoptively transferred into infected semi-immune mice on day 2 or day 4 after patency, respectively. Survival was monitored by flow cytometry and is expressed as a percentage change in total number of transferred RBCs; n = 6 ± SEM. The inset shows the percentage change in Hb (▲) in semi-immune mice over the same period of days after patency. (B) Comparison of rate of the survival of naive CSFE-labeled (●), naive biotin-labeled (○), and resident host biotin-labeled (■) cells in semi-immune mice. Values are expressed as percentage change in the total number of cells present from day 4 after patency (100%). n = 6 ± SEM. (C) RBCs from (i) noninfected naive mice, (ii) nonanemic semi-immune mice on the day of patency, and (iii) anemic semi-immune mice on day 4 after patency were biotinylated in vivo and adoptively transferred into naive recipients. Survival was monitored by flow cytometry. Plots show circulating RBC populations in the recipients on the day of transfer (day 0) and day 6 after transfer. Survival rate values show the mean percentage survival of cells from each donor group on day 6 after transfer. n = 5 ± SEM.

(Figure 3C) when parasites were no longer detectable in circulation. This suggests that the mechanism driving RBC clearance remained active after patent parasitemias had resolved.

Accelerated turnover of transfused RBCs during SMA

We sought to compare the clearance rates of freshly transfused RBCs with those of resident host RBCs during SMA. Differentially labeled RBCs from naive noninfected mice were transfused into infected semi-immune mice at day 2 after patency (CFSE-labeled) and day 4 after patency (biotin-labeled). Transfer populations were limited to 5% to 10% of circulating cells to minimize the impact on recipient Hct. To control for the efficiency of RBC transfer between groups, cell survival was expressed as the percentage change in the total number of transferred cells in circulation, as monitored by flow cytometry (Figure 4A). When transferred into noninfected naive controls, CFSE- and biotin-labeled RBCs showed survival rates of 66% and 65%, respectively, by day 14 after transfer (not shown). However, when transferred into SMA mice on day 2 after patency, naive CSFE-labeled RBCs underwent rapid clearance with a 68% decrease in cell number within 48 hours of transfer. This corresponded with the period of anemic crisis of the host (Figure 4A, inset). Naive biotin-labeled cells were then transferred into semi-immune mice on day 4 after patency, the point of minimum Hb. These transferred cells were 50% cleared from circulation 4.5 days after transfer, corresponding to day 8.5 after patency. The clearance rates of naive cells transferred on days 2 and 4 after patency were compared with the clearance rate of labeled host RBCs (Figure 4B). Cell survival is expressed as the percentage change of total cell number from day 4 after patency onward. Clearance rates were not significantly different, regardless of whether RBCs were long-term host residents or had been transferred at different time points after patency. Thus during SMA, RBCs transferred from naive donors were cleared concurrently with resident host cells, and the length of time RBCs were exposed to the host environment in infected anemic animals did not influence cell survival rates.

Normal survival of RBCs from SMA donors in resting recipients

Next, we sought to determine whether RBCs taken from semi-immune mice undergoing SMA were subject to accelerated clearance when transferred into naive resting recipients. RBCs were taken from 3 donor groups: noninfected, nonanemic controls (Figure 4Ci); infected semi-immune mice on the day of patency (Figure 4Cii); and infected semi-immune mice 4 days after patency, during the peak of anemic crisis (Figure 4Ciii). Donor RBCs were biotinylated in vivo, harvested, transferred into naive recipients, and traced by flow cytometry to determine cell survival. RBCs from the 3 donor groups showed no difference in their rates of clearance in naive recipients (Figure 4C). On day 6 after transfer, the survival of RBCs was 83%, 84%, and 83% for control, day of patency, and day 4 after patency donors, respectively ($P = .46$). Therefore, RBCs from SMA donors displayed normal survival in naive resting recipients, suggesting no intrinsic changes to the RBCs themselves. Thus, alterations to the host environment may be responsible for the accelerated RBC turnover clearance during SMA.

Depletion of phagocytic cells alleviates SMA

The contribution of host phagocytic cells to the development of SMA was investigated by liposome-encapsulated clodronate depletion of macrophage populations.[22] Clodronate depletion of phagocytic macrophages resulted in significantly higher peak parasitemias, 6.3% ± 0.9% by day 4 after patency versus 2.5% ± 0.7% for PBS-treated controls ($P < .001$) (Figure 5A). Thus, macrophages contribute significantly to effector mechanisms controlling *P berghei* parasitemia in semi-immune mice. However, despite increased parasite burdens, the depletion of phagocytic cells alleviated the severity of SMA, with higher Hb levels on day 4 ($P < .001$) and day 6 ($P < .01$) after patency (Figure 5B). Protection from SMA after phagocyte depletion was transient, with the anemic crisis delayed by 4 days. The Hb profile of mice depleted of phagocytic macrophages was significantly different to that of

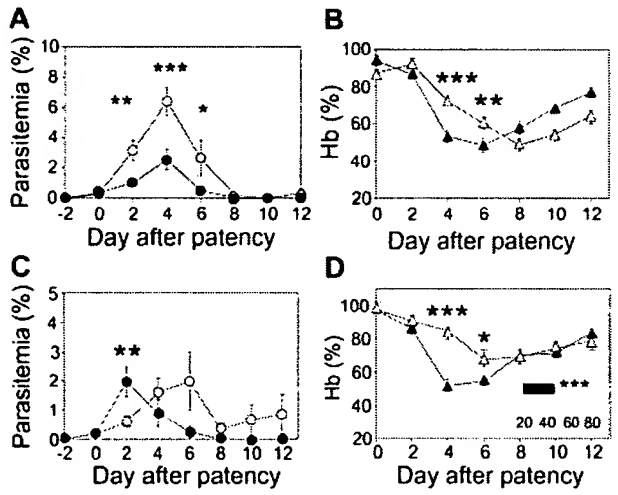

Figure 5. Depletion of immune effector cells alleviates SMA in semi-immune mice. (A) Parasitemia and (B) Hb levels, in phagocyte-depleted semi-immune mice (open symbols) and control semi-immune mice (closed symbols), normalized to the day of patency. n = 9 ± SEM. (C) Parasitemia and (D) Hb levels, in CD4+ cell–depleted (open symbols) and control (closed symbols) mice, normalized to day of patency. Mean individual minimal Hb levels for CD4+ cell–depleted (open bars) and controls (closed bars) are shown in the inset. n = 8 ± SEM for the CD4+ T-cell–depleted and n = 7 ± SEM for control groups. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

controls across the entire curve ($P < .001$), implicating this population in the pathogenesis of SMA.

CD4+ T cells contribute to the severity of SMA

As T lymphocytes are key regulators of macrophage function, we sought to determine whether CD4+ T cells play a role in the etiology of SMA of low parasite burden. CD4+ T cells of infected semi-immune mice were depleted by in vivo administration of lytic monoclonal antibody from the day of patency. Controls received a nonlytic antibody of the same isotype. The average peak parasitemias in the 2 groups were similar at 2.0% (Figure 5C). Depletion of CD4+ T cells modestly influenced the kinetics of infection with a delay in peak parasitemia and an inability to completely resolve infection (Figure 5C). Despite this, mice depleted of CD4+ T cells showed delayed kinetics in the onset of the anemic crisis (Figure 5D) with significantly higher Hb levels on day 4 ($P < .001$) and day 6 ($P < .05$) after patency. Across the cohort, anemia was significantly alleviated in CD4-depleted mice with average minimum Hb levels of 65% of baseline compared with 47% in controls ($P < .001$; Figure 5D, inset).

Discussion

It has long been proposed that the degree of anemia experienced during clinical malaria infection cannot be completely accounted for by the loss of RBCs due to the level of parasitemia, leading to the idea that SMA arises in part from the destruction of nonparasitized cells.[4,16] Clinical and experimental malaria literature has acknowledged that acute infections in naive mouse models do not accurately recapitulate this phenomenon, due to the high burdens of parasitemia experienced.[23,24] The novel semi-immune BALB/c model developed in this study, however, shows the development of SMA at low parasite burden. Following normalization to the day of patency, hematologic profiles displayed highly correlated and predictable parameters of infection, anemia, and recovery, thus establishing the semi-immune BALB/c mouse as a robust and amenable experimental model of SMA.

The profiles of SMA from individual semi-immune BALB/c mice bear a striking resemblance to those of semi-immune *Aotus* monkeys challenged with *P falciparum*,[14] with the onset of anemia at low parasite burden closely following the development of a patent infection. Of importance, similar profiles of SMA at low parasite burden are seen in both experimental *P falciparum* infections in naive humans[4] and in naturally acquired infections in endemic regions.[3] SMA of low parasite burden is consistently observed in multiple host-*Plasmodium* combinations: semi-immune mouse-*berghei* (Figure 1D), naive rat-*berghei* (Figure 1E), semi-immune *Aotus-falciparum*,[14,15] semi-immune and naive human-*falciparum*,[4] and naive human-*vivax*,[16] suggesting that the mechanisms of pathogenesis are conserved across taxa. The conclusion of Jakeman et al[4] and Egan et al[14] regarding the pathogenesis of SMA in *P falciparum* infections of *Aotus* and humans was that anemia must result from the accelerated destruction of uRBCs; however, no experimental evidence of this phenomenon has been presented. The semi-immune BALB/c mouse model and the naive Wistar rat model of SMA at low parasite burden provide experimental demonstration of the accelerated destruction of uRBCs during malaria infection.

In the semi-immune mouse model, severe anemia develops in response to malaria infection at low parasite burden in the presence of appropriate erythropoietic responses. Therefore, this model allows assessment of the contribution of increased RBC destruction to the development of anemia, as confounding factors such as decreased RBC production and RBC loss due to parasite invasion have been minimized. In semi-immune mice, the clearance of RBCs from circulation accounted for the severity of Hb loss at the onset of SMA; however, accelerated turnover of RBCs continued even while Hb levels were restored (Figure 3A). The extent of RBC loss during the anemic crisis included the entire initial blood compartment of 9×10^9 cells and may indeed be greater due to ongoing RBC loss during the recovery phase. In semi-immune mice, cumulative parasitemias were calculated to be approximately 3% of circulating cells over the course of infection, which suggests a ratio of more than 30:1 loss of nonparasitized–parasitized RBCs during SMA. Previously calculated ratios of approximately 10:1 during human SMA[4,5] may therefore be underestimates, as RBC losses were deduced from net minimum Hb values without adjusting for ongoing cell turnover during recovery. There is evidence for continuing RBC turnover during human SMA, as recovery rates of Hb are reduced compared with anemia due to other causes (eg, blood loss trauma),[5] and anemia is known to persist after clearance of parasites.[25] This phenomenon was recapitulated in the murine model, with delayed recovery from SMA compared with PHZ-induced hemolysis (Figure 2B). Together, the data support a protracted period after peak anemia, in which clearance of RBCs continues in the absence of a patent infection, accounting for the impeded return to baseline Hb levels during SMA.

Accelerated RBC turnover in human SMA is proposed to result from acquired changes to the surface or structure of noninfected RBCs that target them for destruction either by intravascular hemolysis or immune-mediated clearance.[6] High levels of intravascular hemolysis induce hematuria in both mice[26] and humans,[27] but hematuria is uncommon in clinical malaria infections in endemic areas[28] and was not observed in the SMA models elucidated here. Hb balance studies of malaria patients suggest that extravascular clearance is the major mechanism of RBC destruction during infection,[29] and therefore it is unlikely that intravascular hemolysis is a major contributory mechanism to SMA at low parasite burden.

RBC transfer experiments revealed that cells fated for destruction in hosts experiencing SMA did not express sufficient changes to cause accelerated clearance in a normal resting animal, and, upon transfusion, fresh cells from naive donors were cleared at the same rate as resident RBCs in an SMA host (Figure 4). These findings suggest that changes to target RBCs play a minimal role in the etiology of SMA in semi-immune mice, and, conversely, mice experiencing SMA express a physiologic status sufficient to cause accelerated destruction of RBCs independent of the target cell history. This is consistent with the previously reported lack of detectable modifications such as immunoglobulin or complement deposition on RBCs in the semi-immune *Aotus* model[15] and the lack of a consistent association of positive direct antiglobulin test (DAT) and SMA in humans.[30,31] The survival curves of naive RBCs transferred into infected semi-immune mice showed strong similarities to the survival curves of compatible donor RBCs transferred into the circulation of patients recovering from *P falciparum* and *P vivax* infection.[10,32] These studies demonstrated that during human malaria infection RBCs have a significantly shortened mean cell half-life and that both autologous and compatible donor RBCs experience clearance from circulation at equal rates following successful treatment with antimalarial drugs. This provides a clinical validation of the processes occurring in rodent models and the combined transfer data suggest that changes extrinsic to the RBCs themselves may be responsible for their accelerated clearance. Further to this, transfusion studies conducted during the treatment of anemic Kenyan children showed that 25% of patients receiving blood transfusions experienced rises in Hb of less than 20 g/L (2 g/dL), and that 25% of severely anemic children maintained Hb less than 50 g/L (5 g/dL) after transfusion.[33] A continuing destruction of RBCs, independent of their source, may account for exacerbated anemia in the face of appropriate therapeutic intervention and explain the relatively poor ability of transfusion to elevate Hb levels in these patients.

The significant alleviation of SMA by depletion of host $CD4^+$ T cells or phagocytic cells (Figure 5) establishes the contribution of immune mechanisms to the pathogenesis of SMA. Hb levels were 35% greater in phagocyte-depleted and 64% greater in $CD4^+$-depleted semi-immune mice on day 4 after patency compared with nontreated controls. The data presented here strongly support the notion that, akin to other life-threatening malarial pathologies,[17] SMA is mediated in part by immunopathogenic mechanisms. In particular, we propose that the destruction of uRBCs may result from a hyperactivated phagocytic system. Phagocytosis of noninfected RBCs has been documented during human infections,[34,35] and hyperphagocytosis has been implicated in malarial thrombocytopenia.[36] Constitutive or basal homeostatic RBC clearance is predominantly mediated by splenic red pulp macrophages, and increased recruitment or activation of this population during malaria infection may accelerate splenic RBC clearance. SMA in humans is associated with high serum levels of neopterin, a marker of macrophage activation, induced particularly by IFN-γ.[37] These observations have led to the proposal that SMA has an inflammatory etiology.[38] Counterregulatory T_H2 cytokines such as IL-4[37] and IL-10[39,40] are inversely associated with malarial anemia, suggesting that a loss of host cytokine regulation contributes to the severity of disease. Thus, the accelerated turnover of RBCs during SMA may be regulated by diverse factors controlling macrophage phagocytic activity and recruitment, such as host $CD4^+$ T cells, cytokine/chemokine cascades, and bioactive parasite products such as hemozoin[41] and glycosylphosphatidylinositol.[42,43] Significantly, adoptive transfer of parasite-specific, T_H1 $CD4^+$ T-cell lines promotes anemia during naive *P berghei* infections,[44] and this may relate to the capacity of $CD4^+$ T cells to up-regulate macrophages. As SMA is pronounced in *Aotus* immunized with *P falciparum* antigens,[14,15] blood-stage vaccines that suppress parasitemias may not necessarily protect against the development of anemia. Whether T-cell priming by these experimental vaccines promotes SMA is not clear. Rodent models of SMA may provide useful preclinical test systems to assess further the efficacy of vaccines in prevention or promotion of this disease state.

SMA does not result in fatality in the animal models presented here, as physiologically appropriate reticulocytosis eventually restored Hb to basal levels. In rodents, the erythropoietic response to acute anemia occurs predominantly in the spleen, and it is likely that extramedullary erythropoiesis facilitates the recovery from SMA in these models. However, in humans, SMA could be exacerbated or even fatal should erythropoietic suppression prevent adequate reticulocyte compensation. *P falciparum* infection is often associated with impaired erythropoietic responses, with patients displaying suboptimal reticulocyte levels for the degree of malarial anemia experienced.[45,46] Malaria-induced erythropoietic suppression has been studied in acute infections of experimental animal models, which have provided insights into the mechanisms of decreased RBC production during infection.[47-50] During malaria infection, a decreased responsiveness to adequate levels of erythropoietin may lead to suppressed development of erythroid precursors, resulting in an inappropriately low level of production of new RBCs, as recently reviewed.[24] Therefore, during SMA, erythropoietic suppression may overlap with the accelerated destruction of RBCs, leading to an exacerbated syndrome and fatality. The quantitative contribution of each mechanism may vary across differing clinical and epidemiologic settings, contributing to varying patterns of malarial pathology.[51] As rodents are tractable models amenable to experimental perturbation, it may now be possible in both acute and semi-immune infections to investigate more closely the multiple host and parasite factors proposed to influence these 2 contributory mechanisms to SMA in vivo. These findings thus extend further the use of rodent malarias as models for disease processes in humans.

References

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature. 2005; 434:214-217.
2. Murphy SC, Breman JG. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am J Trop Med Hyg. 2001;64:57-67.
3. Bojang KA, Van Hensbroek MB, Palmer A, Banya WA, Jaffar S, Greenwood BM. Predictors of mortality in Gambian children with severe malaria anaemia. Ann Trop Paediatr. 1997;17:355-359.
4. Jakeman GN, Saul A, Hogarth WL, Collins WE. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology. 1999; 119(pt 2):127-133.
5. Price RN, Simpson JA, Nosten F, et al. Factors contributing to anemia after uncomplicated falciparum malaria. Am J Trop Med Hyg. 2001;65: 614-622.
6. Ekvall H. Malaria and anemia. Curr Opin Hematol. 2003;10:108-114.
7. Das BS, Nanda NK. Evidence for erythrocyte lipid peroxidation in acute falciparum malaria. Trans R Soc Trop Med Hyg. 1999;93:58-62.
8. Dondorp AM, Angus BJ, Chotivanich K, et al. Red blood cell deformability as a predictor of anemia in severe falciparum malaria. Am J Trop Med Hyg. 1999;60:733-737.
9. Goka BQ, Kwarko H, Kurtzhals JA, et al. Complement binding to erythrocytes is associated with macrophage activation and reduced haemoglobin in Plasmodium falciparum malaria. Trans R Soc Trop Med Hyg. 2001;95:545-549.
10. Looareesuwan S, Merry AH, Phillips RE, et al.

Reduced erythrocyte survival following clearance of malarial parasitaemia in Thai patients. Br J Haematol. 1987;67:473-478.

11. Layez C, Nogueira P, Combes V, et al. Plasmodium falciparum rhoptry protein RSP2 triggers destruction of the erythroid lineage. Prepublished on July 26, 2005, as DOI 10.1182/blood-2005-04-1574. (Now available as Blood. 2005;106:3632-3638.
12. Langhorne J, Quin SJ, Sanni LA. Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. Chem Immunol. 2002;80:204-228.
13. WHO. Severe falciparum malaria: World Health Organization, Communicable Diseases Cluster. Trans R Soc Trop Med Hyg. 2000;94(suppl 1):S1-S90.
14. Egan AF, Fabucci ME, Saul A, Kaslow DC, Miller LH. Aotus New World monkeys: model for studying malaria-induced anemia. Blood. 2002;99:3863-3866.
15. Jones TR, Stroncek DF, Gozalo AS, et al. Anemia in parasite- and recombinant protein-immunized aotus monkeys infected with Plasmodium falciparum. Am J Trop Med Hyg. 2002;66:672-679.
16. Collins WE, Jeffery GM, Roberts JM. A retrospective examination of anemia during infection of humans with Plasmodium vivax. Am J Trop Med Hyg. 2003;68:410-412.
17. Schofield L, Grau GE. Immunological processes in malarial pathogenesis. Nat Rev Immunol. 2005;5:722-735.
18. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods. 1994;174:83-93.
19. Elso CM, Roberts LJ, Smyth GK, et al. Leishmaniasis host response loci (lmr1–3) modify disease severity through a Th1/Th2-independent pathway. Genes Immun. 2004;5:93-100.
20. Mock DM, Lankford GL, Widness JA, Burmeister LF, Kahn D, Strauss RG. Measurement of red cell survival using biotin-labeled red cells: validation against 51Cr-labeled red cells. Transfusion. 1999;39:156-162.
21. Strauss RG, Mock DM, Johnson K, et al. Circulating RBC volume, measured with biotinylated RBCs, is superior to the Hct to document the hematologic effects of delayed versus immediate umbilical cord clamping in preterm neonates. Transfusion. 2003;43:1168-1172.
22. van Rooijen N, Kors N, Kraal G. Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. J Leukoc Biol. 1989;45:97-104.
23. Looareesuwan S, Ho M, Wattanagoon Y, et al. Dynamic alteration in splenic function during acute falciparum malaria. N Engl J Med. 1987;317:675-679.
24. Chang KH, Stevenson MM. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. Int J Parasitol. 2004;34:1501-1516.
25. Camacho LH, Gordeuk VR, Wilairatana P, Pootrakul P, Brittenham GM, Looareesuwan S. The course of anaemia after the treatment of acute, falciparum malaria. Ann Trop Med Parasitol. 1998;92:525-537.
26. Criswell KA, Sulkanen AP, Hochbaum AF, Bleavins MR. Effects of phenylhydrazine or phlebotomy on peripheral blood, bone marrow and erythropoietin in Wistar rats. J Appl Toxicol. 2000;20:25-34.
27. Arese P, De Flora A. Pathophysiology of hemolysis in glucose-6-phosphate dehydrogenase deficiency. Semin Hematol. 1990;27:1-40.
28. Phillips RE, Looareesuwan S, Warrell DA, et al. The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. Q J Med. 1986;58:305-323.
29. Ekvall H, Arese P, Turrini F, et al. Acute haemolysis in childhood falciparum malaria. Trans R Soc Trop Med Hyg. 2001;95:611-617.
30. Abdalla S, Weatherall DJ. The direct antiglobulin test in P. falciparum malaria. Br J Haematol. 1982;51:415-425.
31. Merry AH, Looareesuwan S, Phillips RE, et al. Evidence against immune haemolysis in falciparum malaria in Thailand. Br J Haematol. 1986;64:187-194.
32. Looareesuwan S, Davis TM, Pukrittayakamee S, et al. Erythrocyte survival in severe falciparum malaria. Acta Trop. 1991;48:263-270.
33. English M, Ahmed M, Ngando C, Berkley J, Ross A. Blood transfusion for severe anaemia in children in a Kenyan hospital. Lancet. 2002;359:494-495.
34. Abdalla SH. Peripheral blood and bone marrow leucocytes in Gambian children with malaria: numerical changes and evaluation of phagocytosis. Ann Trop Paediatr. 1988;8:250-258.
35. La Raja M. Erythrophagocytosis by peripheral monocytes in Plasmodium falciparum malaria. Haematologica. 2002;87:EIM14.
36. Erhart LM, Yingyuen K, Chuanak N, et al. Hematologic and clinical indices of malaria in a semi-immune population of western Thailand. Am J Trop Med Hyg. 2004;70:8-14.
37. Biemba G, Gordeuk VR, Thuma PE, Mabeza GF, Weiss G. Prolonged macrophage activation and persistent anaemia in children with complicated malaria. Trop Med Int Health. 1998;3:60-65.
38. Biemba G, Gordeuk VR, Thuma P, Weiss G. Markers of inflammation in children with severe malarial anaemia. Trop Med Int Health. 2000;5:256-262.
39. Kurtzhals JA, Adabayeri V, Goka BQ, et al. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. Lancet. 1998;351:1768-1772.
40. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V. A low interleukin-10 tumor necrosis factor-alpha ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. J Infect Dis. 1999;179:279-282.
41. Urban BC, Roberts DJ. Malaria, monocytes, macrophages and myeloid dendritic cells: sticking of infected erythrocytes switches off host cells. Curr Opin Immunol. 2002;14:458-465.
42. Schofield L, Hewitt MC, Evans K, Siomos MA, Seeberger PH. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. Nature. 2002;418:785-789.
43. Schofield L, Novakovic S, Gerold P, Schwarz RT, McConville MJ, Tachado SD. Glycosylphosphatidylinositol toxin of Plasmodium up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. J Immunol. 1996;156:1886-1896.
44. Hirunpetcharat C, Finkelman F, Clark IA, Good MF. Malaria parasite-specific Th1-like T cells simultaneously reduce parasitemia and promote disease. Parasite Immunol. 1999;21:319-329.
45. Abdalla S, Weatherall DJ, Wickramasinghe SN, Hughes M. The anaemia of P. falciparum malaria. Br J Haematol. 1980;46:171-183.
46. Srichaikul T, Panikbutr N, Jeumtrakul P. Bone-marrow changes in human malaria. Ann Trop Med Parasitol. 1967;61:40-51.
47. Villeval JL, Lew A, Metcalf D. Changes in hemopoietic and regulator levels in mice during fatal or nonfatal malarial infections: I, erythropoietic populations. Exp Parasitol. 1990;71:364-374.
48. Chang KH, Tam M, Stevenson MM. Modulation of the course and outcome of blood-stage malaria by erythropoietin-induced reticulocytosis. J Infect Dis. 2004;189:735-743.
49. Chang KH, Tam M, Stevenson MM. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. Blood. 2004;103:3727-3735.
50. Sexton AC, Good RT, Hansen DS, et al. Transcriptional profiling reveals suppressed erythropoiesis, up-regulated glycolysis, and interferon-associated responses in murine malaria. J Infect Dis. 2004;189:1245-1256.
51. Marsh K. Malaria: a neglected disease? Parasitology. 1992;104(suppl):S53-S69.

Although it is now possible to isolate lymphatic endothelial cells, in vitro systems to study their development were lacking. Embryonic stem (ES) cells are extremely useful to study cellular differentiation processes involved in early-stage organ development because they have the amazing potential to differentiate into a variety of different cell types in vitro, including the cellular constituents of the cardiovascular system.[4] Such experiments were instrumental for studying the differentiation of blood-vessel endothelial cells from mesodermal progenitors.

In this issue of *Blood,* Liersch and colleagues report the induction of lymphatic endothelial-cell differentiation in ES cell–derived embryoid bodies. These cells were positive for the panendothelial marker CD31/PECAM-1 and the lymphatic endothelial markers Prox-1 and LYVE-1 (see figure), but negative for the blood vascular marker MECA-32. Lymphatic vessels frequently sprouted from blood-vessel structures, consistent with the in vivo observations. Of interest, the addition of certain growth factors known to induce lymphangiogenesis in vivo revealed significant differences in their potential to promote lymphatic endothelial-cell differentiation in vitro: VEGF-C, and, to a lesser extent, VEGF-A, promoted lymphatic vessel formation, whereas basic fibroblast growth factor did not. Whether these differences indicate that lymphangiogenesis in vivo involved indirect effects, or simply reflect limitations of the in vitro system, remains to be clarified.

One interesting question that is not addressed by the study is whether lymphatic endothelium forms solely by sprouting from blood-vessel endothelium, or whether it differentiates also de novo from endothelial progenitor cells, as blood-vessel endothelium does. In other words, is (lymph-) vasculogenesis involved in lymph vessel formation? The authors plan to address this question in the future by time-lapse studies. Thus, the availability of an in vitro system for studying the differentiation of lymphatic endothelium is a major step toward the elucidation of the molecular events that govern the formation of the lymphatic vasculature. ■

REFERENCES

1. Sabin FR. On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. Am J Anat. 1902;1:367-391.
2. Hong YK, Shin JW, Detmar M. Development of the lymphatic vascular system: a mystery unravels. Dev Dyn. 2004;231:462-473.
3. Oliver G, Alitalo K. The lymphatic vasculature: recent progress and paradigms. Annu Rev Cell Dev Biol. 2005;21:457-483.
4. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev. 2005;19:1129-1155.

RED CELLS

Comment on Evans et al, page 1192

Malaria: death and disappearing erythrocytes

John W. Barnwell CENTERS FOR DISEASE CONTROL AND PREVENTION

Severe anemia is the cause of a large proportion, if not a majority, of the 1 to 2 million deaths of African children that are attributed to malaria each year.

One hundred and twenty-five years after the discovery of malaria parasites, remarkably, the mechanism(s) responsible for severe malarial anemia (SMA) syndrome are not well understood. Certainly, anemic states develop in malaria cases exhibiting hyperparasitemia or blackwater fever through intravascular hemolysis. However, these cases represent a very minor portion of the malarial anemia burden encountered in areas of endemic transmission. SMA in Sub-Saharan Africa and other areas of high to moderate malaria transmission occurs in younger children (1-3 years) after prior experience with malaria parasites and often becomes manifest in chronic infections of relatively low parasite burden (< 1%-2% parasitemia).[1]

In most SMA cases, intravascular hemolysis is not apparent and destruction of uninfected erythrocytes is more than 10-fold greater than any lysis caused by parasitization. There also maybe impaired reticulocytosis with failure to replace the red blood cell (RBC) pool even as a portion continues to be lost. Thus, one thesis is that a misfunctioning bone marrow and extravascular removal of nonparasitized RBCs combine to bring about an SMA crisis leading to death if treatment is not provided.[2] However, because of high rates of drug resistance and HIV in African countries, treatments fail and transfusions can be dangerous with their own fatal consequences. Furthermore, despite adequate therapy, transfused RBCs are often rapidly removed despite declining or absent levels of parasitemia.[3] A fuller understanding of SMA etiology could provide for improved treatment protocols, and there is a need for animal models that mimic SMA in human patients to obtain this knowledge.

Evans and colleagues have devised such a model of SMA using *Plasmodium berghei* infections in semi-immune mice and in malaria-naive rats. The rodent models mimic human SMA in that at low parasite burdens hemoglobin levels fall to less than 50% of normal at crisis and are independent of parasite loads but are related to chronicity. Prior studies of SMA in rodent malaria models relied on either fatal nonresolving infections or nonfatal resolving infections that were confounded by high parasitemias and iRBC lysis. Of importance, the investigations of Evans et al indicate that intravascular hemolysis is not a contributory factor to SMA and that there is a near-complete turnover of the blood pool in a week. Further, it is shown that RBCs from naive mice are removed at the same rapid rate in infected mice with SMA with no accelerated clearance in normal mice of RBCs from infected mice, suggesting that changes to RBCs in infected mice to target their removal are not significant factors in SMA. While it has been suspected that changes to the RBC surface such as Ig, complement, or, as recently reported,[4] parasite antigen deposition onto bystander cells might underlie an extravascular clearance mechanism in SMA, demonstration of these modifications has been inconsistent in human SMA.

The SMA model developed by Evans and colleagues does differ from SMA in humans since erythropoiesis in the rodent model does function, producing a strong compensatory reticulocytosis with eventual recovery and survival. In *Plasmodium falciparum*–induced human and nonhuman primate models of malignant tertian SMA there is frequently a lack of compensatory reticulocytosis,[2,5] whether parasites are patent or not, which leads to high

mortality if unchecked. Survival in the rodent SMA model may occur because the spleen in rodents is a major erythropoietic organ, whereas in primates it is not. Alternatively, it may be that rodent SMA models lack certain features of SMA that occur in *P falciparum* infections in humans and its nonhuman primate models. Clearly, continued investigations into SMA mechanisms in both the rodent models and the nonhuman primate models are important to gain fresh insight leading to new therapeutic interventions for SMA.

REFERENCES

1. Reyburn H, Mbatia R, Drakeley C, et al. Association of transmission intensity and age with clinical manifestations and case fatality of severe Plasmodium falciparum malaria. JAMA. 2005;293:1461-1470.
2. Ekvall H. Malaria and anemia. Curr Opin Hematol. 2003;10:108-114.
3. English M, Ahmed M, Ngando C, et al. Blood transfusion for severe anemia in children in a Kenyan hospital. Lancet. 2002;359:494-495.
4. Layez C, Nogueira P, Combes V, et al. Plasmodium falciparum rhoptry protein RSP2 triggers destruction of the erythroid lineage. Blood. 2005;106:3632-3638.
5. Egan AF, Fabucci ME, Saul A, et al. Aotus New World monkeys: model for studying malaria-induced anemia. Blood. 2002;99:3863-3866.

HEMATOPOIESIS

Comment on Yilmaz et al, page 924

Stem cell markers: less is more!

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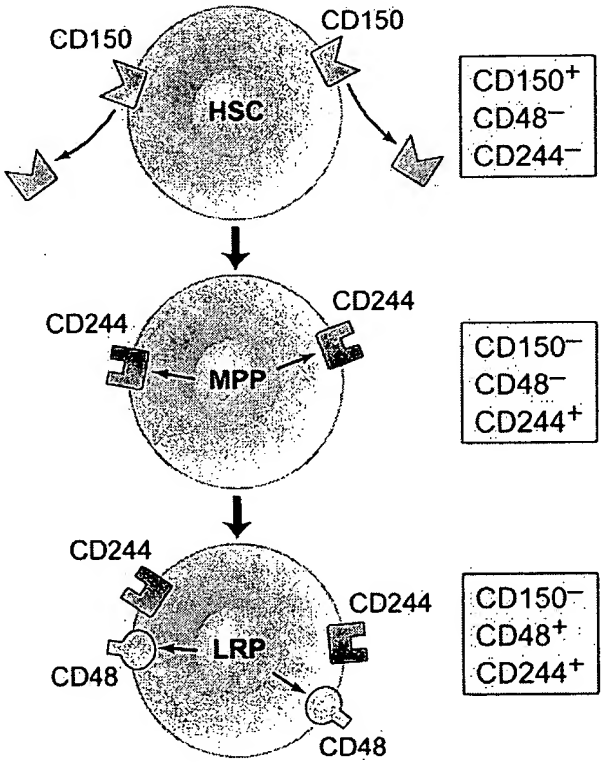
Yilmaz and colleagues present a simplified technique for the prospective identification and purification of hematopoietic stem cells; unlike previous methods, their method is effective in a variety of contexts, including old marrow, mobilized peripheral blood, and recipients of long-term engrafted transplants.

In this issue of *Blood*, Yilmaz and colleagues address a sine qua non of stem cell biology—the prospective identification and purification of viable hematopoietic stem cells. The Morrison lab determined that a simple combination of 2 cell-surface markers, CD150 and CD48, is sufficient to identify all of the long-term repopulating activity from not only young mouse bone marrow, but also, importantly, from bone marrow of old mice, from long-term engrafted radiation chimeras, and from mobilized stem cells found in the spleen. This feat has not been possible with other stem cell purification techniques. In a paper in *Cell* earlier this year,[1] this group detailed the use of members of this SLAM family of cell receptors to characterize specific developmental steps in the stem and progenitor cell hierarchy in bone marrow of young mice and arrived at the simple SLAM "code" for stem cells as $CD150^+$ and $CD48^-/CD244^-$ (see figure).

Heretofore, typical methods have relied on a combination of up to 10 cell-surface markers to characterize hematopoietic stem cells. Despite its difficulty and nuances, this technique has been in successful practice, and has advanced our understanding of stem cell biology, since its original publication in 1988.[2] As interest in hematopoietic stem cells from different aged animals and from different hematopoietic sources has grown, limitations have become apparent, the most significant of which has been the issue of whether the stem cell markers that were used maintained their stem-cell fidelity. In earlier studies, Morrison et al showed that the same technique that yielded a population in which 1 in every 5 cells purified from young bone marrow had long-term engrafting potential yielded dramatically lower purities of *functional stem cells* from other sources.[3,4]

The disparity in apparent purities could be attributable to several possibilities. One is that expression of the traditional panel of markers is altered on cells either during aging, mobilization, or prior transplantation such that the staining profile is no longer inclusive of stem cells and/or is no longer exclusive of contaminating cells. A second possibility is that the requisite steps leading to long-term engraftment—that is, homing of stem cells to the marrow and the maintenance of long-term hematopoiesis—are less efficient in stem cells derived from contexts other than young bone marrow. Compelling data in the present paper demonstrate that the bulk of the disparity is accounted for by the presence of cells in the population identified as stem cells (Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$, $Thy1^{lo}$) that demonstrably lack stem cell function. In contrast, the population of $CD150^+$, $CD48^-$ cells from all hematopoietic sources tested showed dramatically improved stem-cell purities, although not quite to the



SLAM cell-surface markers delineate differentiation steps in early hematopoiesis. Originating with pluripotent hematopoietic stem cells (HSCs), differentiation steps include multipotent progenitor cells (MPPs) and lineage-restricted progenitor cells (LRPs). Each is characterized by a different complement of SLAM markers: HSCs are $CD150^+CD48^-CD244^-$; MPPs are $CD150^-CD48^-CD244^+$; LRPs are $CD150^-CD48^+CD244^+$. It should be noted that CD48 is a ligand for CD244, thus $CD150^+CD48^-$ is sufficient to distinguish HSCs from MPPs and LRPs. Illustration by Kenneth Probst.

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Pathogenesis of Cerebral Malaria: Recent Experimental Data and Possible Applications for Humans

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INTRODUCTION

Malaria is still the world's most important parasitic disease and is responsible for the death of more people than any other communicable disease except tuberculosis. According to World Health Organization estimates (61), between 300 million and 500 million people are infected with malaria every year. The disease is a public health problem in more than 90 countries, which are home to some 2,400 million people, 40% of the world's population. More than 90% of all malaria cases are in sub-Saharan Africa. Two-thirds of the remainder are concentrated in six countries, India, Brazil, Sri Lanka, Afghanistan, Vietnam, and Colombia, in decreasing order of prevalence. Mortality due to malaria is in the range of 1.5 million to 2.7 million deaths per year. Deaths occur mostly among young children in Africa, especially in remote rural areas with poor access to health services. Cerebral malaria (CM) is the most severe complication and the major cause of death. In some reports, CM accounts for up to 10% of all cases of *Plasmodium falciparum* malaria in hospitalized persons and for 80% of fatal cases.

The classic clinical presentation of malaria consists of bouts of fever accompanied by other symptoms such as headache, malaise, nausea, muscular pains, or mild diarrhea, often mistaken for influenza or gastrointestinal infection. Details of the diagnosis, outcome in different types of patients, and treatment recommendations have been extensively reviewed (118), as have the socioeconomic consequences of the malaria burden worldwide (72). Although various hypotheses have been proposed and some progress has been made using in vitro as well as in vivo models, the mechanisms of CM pathogenesis remain incompletely understood and are the subject of a continuing debate (9, 14, 32).

To investigate the pathogenesis of CM, several animal models have been established in which animals are infected with erythrocytes parasitized by various types of *Plasmodium*. Although these animal models do not exactly reproduce the human disease, they nevertheless exhibit some similarities to human CM, such as clinical signs of nervous system dysfunction and cerebral pathology. An elegant and exhaustive review on human CM pathology has been published recently (109). However, the assertion that "there is no model for CM," based on the fact that murine CM shows leukcocyte sequestration, can be modulated, since the presence of leukcocyte sequestration in human CM, at least in pediatric patients, is now well substantiated (32, 37). Conversely, parasitized red blood cell (pRBC) sequestration also occurs in murine CM, although in a less prominent fashion than in humans (42a).

Several observations from the study of experimental CM have been extended and confirmed in human disease. First, cytokine overproduction was detected during experimental CM and was found to contribute to brain vascular pathology. Tumor necrosis factor TNF (33, 34) and gamma interferon (IFN-γ) were shown to be important mediators in the pathogenesis of CM. Second, helper T lymphocytes play a significant role in the development of murine CM (25, 38). In response to

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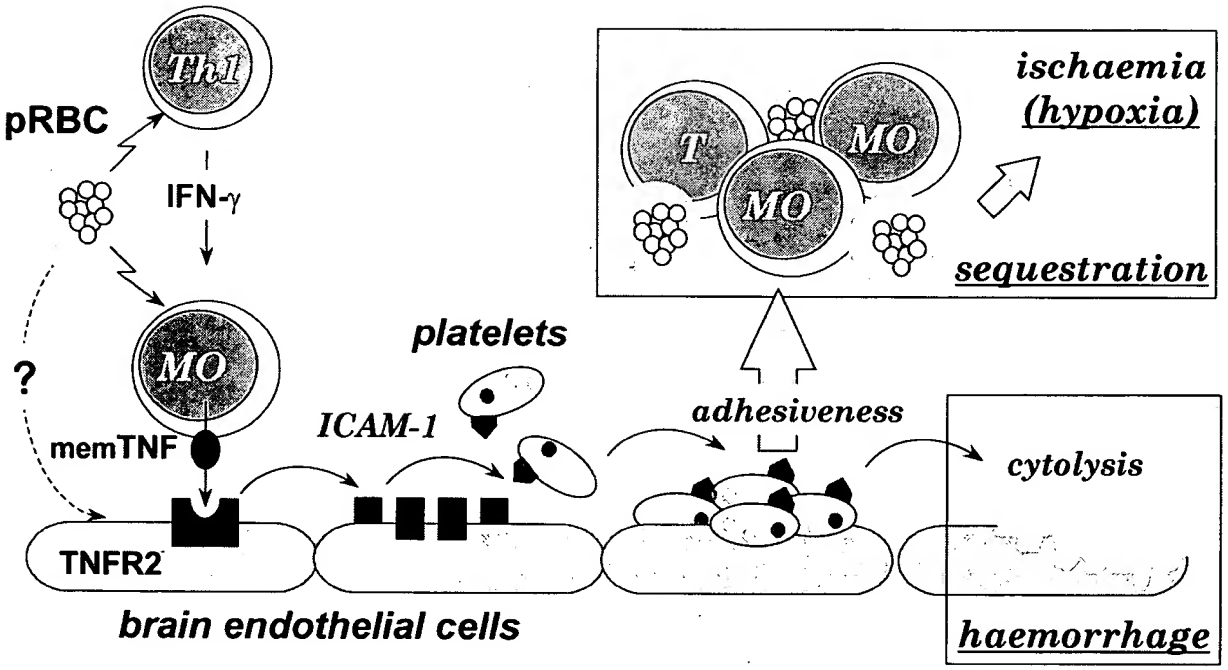

FIG. 1. Importance of other blood cells in the modulation of pRBC binding in the pathogenesis of CM. The malarial parasite (pRBC) stimulated the host immune response, notably an expansion of Th1 clones, leading to overproduction of IFN-γ. Apart from upregulating some potential receptors, such as CD36, IFN-γ stimulates monocytes to produce soluble TNF (solTNF) and to express higher levels of the transmembrane form of the cytokine (memTNF). Both forms, but particularly the memTNF via an interaction with TNFR2 expressed in increased amounts, cause an upregulation of ICAM-1 on brain endothelial cells. In turn, high levels of ICAM-1 cause platelets to adhere and fuse to brain endothelial cells, with at least two important functional consequences: an increased adhesiveness for pRBC (via CD36) and leukocytes (via LFA-1, P-selectin, etc.), responsible for vessel obstruction, ischemia and possible neuronal dysfunction, and a potentiation of endothelial killing by TNF, leading to vessel disruption and brain hemorrhages.

malaria parasites, the host undergoes a Th1 rather than a Th2 response, in which susceptibility to CM is favored over resistance (11, 16). Third, cytokine-induced phenotypic changes of brain microvascular endothelial cells (MVEC) indeed represent a key event in the sequestration of both PRBC and parasitized leukocytes (22, 39, 85, 110).

Several recent results, from in vivo studies using gene-deficient mice and from in vitro studies using isolated brain MVEC led us to address other mechanisms of experimental CM. These results included (i) an unexpected role of platelets in TNF-induced microvascular pathology, (ii) protection from CM in TNF receptor p75-deficient mice but not p55-deficient mice, and (iii) the finding that brain MVEC derived from CM-susceptible and CM-resistant mice exhibit differential responsiveness to the cytokines TNF and IFN-γ. In this review, we will focus on these findings and their possible meaning in terms of pathogenic mechanisms and will discuss their applicability to human CM. The importance of other blood cells in the modulation of pRBC binding in the pathogenesis of CM is illustrated in Fig. 1.

IN VIVO MODELS OF CEREBRAL MALARIA

Experimental animals models cannot reproduce all the features of human diseases; this is particularly true for CM. However, in CM the altered cells that play a pivotal role in human and experimental lesions are the same (the brain MVEC). In addition, the similarities between defined malaria antigens in rodent and human parasites and between immune response pathways in mice and humans justify the use of models. The concepts defined in experimental conditions might lead to further investigations in the human disease. Several animal models of CM have been established, each of which exhibits specific pathological characteristics depending on the malaria parasites and animal strains employed (Table 1).

The *Plasmodium yoelii* model has an advantage over the *Plasmodium berghei* ANKA (PbA) model in including an obvious pRBC sequestration. However, as discussed above, this cannot be taken as an argument to claim superiority for this

TABLE 1. Examples of in vivo models used for the study of CM

Malaria parasite	Animal	Characteristic	Reference(s)
P. yoelii 17XL	Swiss mice	pRBC sequestration	48, 123
P. berghei ANKA	CBA/Ca mice	Leukocyte sequestration	38, 63, 74, 87
	CBA/T6 mice		73
	DBA/2 mice	Nonfatal CM	73
	C57BL/6 mice		46
P. berghei K173	C57BL/6 mice		15, 19, 20
P. berghei NK65	CBA/Ca mice		117
P. berghei ANKA	Hamster		87
P. berghei NK65	WM/M rat		45
P. knowlesi	Rhesus monkey		105
P. fragile	Rhesus monkey		27
P. coatneyi	Rhesus monkey		1, 101
P. falciparum	Saimiri monkey	pRBC sequestration	106
P. falciparum	SCID mice		120

model (48), since leukocytes are also sequestered in human CM. The leukocyte sequestration seen in the various murine models or in humans is never accompanied by transmigration, such as in experimental autoimmune encephalitis, so it can hardly be called inflammation (46). Monkey models of CM exhibit pathological changes very close to those of human CM, especially since they can reproduce the sequestration of pRBC and brain vascular complications such as hemorrhages. Problems with the monkey model include the facts that the time point of CM onset is difficult to determine and that the expected incidence of this syndrome is low and variable whereas it is high and reproducible in mice. In spite of the cost and the lack of genetically modified animals, which are other limitations, the monkey models represent invaluable tools for the study of pRBC sequestration in vivo.

P. BERGHEI ANKA MODEL

Because of its high degree of reproducibility, easily manageable characteristics, brain histopathology (which is now validated further by recent observations in human CM [see "Perspectives" below]), and relevant clinical expression and the availability of susceptible and resistant strains, we have focused our attention on the neurovascular pathology induced by PbA (originally described by J. M. Bafort, Antwerp, Belgium [4]) asexual blood stages. The PbA model consists of a neurological syndrome occurring 6 to 14 days after infection and with a cumulative mortality of about 90% (33). Parasitemia at the time of death is consistently low. The neurological manifestations include hemi- or paraplegia, deviation of the head, tendency to roll over on stimulation, ataxia, and convulsions. The remaining 10% of infected CM-susceptible mice eventually die during week 3 or 4 of infection, with severe anemia and hyperparasitemia and without neurological signs (33).

Susceptible versus Resistant Mice

The reason why only a small percentage of the human population infected with malaria develops the neurological complication is still unclear. One of the features of the PbA model is to allow studies of the genetic control of susceptibility to CM. Following PbA infection, various mouse strains also exhibit a differential responsiveness to the malaria parasite. Some strains are highly susceptible to the development of CM, while others are resistant, in spite of identical levels of parasitemia during the acute phase of the infection (Table 2) (G. E. Grau et al., unpublished results).

These data clearly show that genes outside the H-2 complex are involved in the genetic control of susceptibility to CM. Other factors include the capacity to produce IFN-γ in response to malarial antigens (see below).

Brain Histopathology

The histopathology of experimental CM varies according to parasite-host combinations. The differential pathological changes in animal models were found to be related to different malaria parasites. For example, CBA mice clearly exhibit a brain vascular pathology when infected with PbA (33) but not with *P. yoelii* (33) or *P. vinckei* (13). However, *P. vinckei* causes other features of severe falciparum malaria. *P. yoelii* 17XL-infected Swiss mice show a significant sequestration of pRBC

TABLE 2. Mouse strain susceptibility to PbA-induced CM

Strain[a]	*H-2* type	No. of mice	Cumulative incidence of CM (%)	Parasitemia on day 7[b]
CBA/Ca	*k*	>500	90	8.0 ± 3.4
CBA/HN	*k*	10	70	10.2 ± 3.3
C57BL/10	*b*	30	80	8.5 ± 3.2
DBA/1	*q*	30	80	17.0 ± 3.0
NMRI		30	90	6.5 ± 7.0
SJL/J	*s*	20	95	28.0 ± 6.0
BALB/c	*d*	>100	0	10.5 ± 4.5
C3H/HeJ	*k*	30	0	17.0 ± 5.2
C3H/HeN	*k*	10	0	16.0 ± 4.5
DBA/2	*d*	30	0	15.0 ± 3.5
(NZB×NZW)F1	*z*	10	0	6.3 ± 4.2

[a] In addition to these strains studied in our laboratory, the A/J mouse has recently been reported to be resistant to CM on PbA infection (46).
[b] Number of red blood cells which are parasitized (values are percentages, and standard deviations are shown).

(123). In rhesus monkeys, *P. falciparum* infection is not associated with a sequestration of pRBC, whereas this sequestration does occur during *P. fragile* infection. Conversely, pathological changes induced by a given parasite may vary among different mouse strains. It has been shown that PbA-infected CBA mice develop a fatal cerebral malaria (33, 38, 74, 87) whereas DBA/2 mice develop a nonfatal cerebral syndrome (73) and BALB/c mice do not develop any cerebral pathology (33).

A particularly detailed analysis of brain histopathology has been performed with the CBA/T6 model (73). When inoculated with PbA, these mice exhibited cerebral symptoms and died from cerebral malaria 6 to 8 days after infection whereas DBA/2J mice developed (around day 6 to 9) a nonfatal CM, with milder cerebral symptoms and died between days 15 and 22 from other malaria-related complications. When inoculated with *P. berghei* K173, these mouse strains did not develop cerebral malaria. These mouse-parasite strain combinations were used, in conjunction with the retinal whole-mount technique, to elucidate factors critical in the pathology of murine CM. CBA/T6 mice infected with PbA (PbA-CBA mice) demonstrated mild changes in vascular permeability as early as 2 to 3 days before the appearance on day 5 of cerebral symptoms, whereas mice with noncerebral malaria did not show any vascular permeability changes until the very late stage of the disease (days 14 to 22). In PbA infections, progressive deterioration of endothelial barrier properties, demonstrated by Evans' Blue leakage both generally and from specific focal areas, and developing monocytosis and adherence of mononuclear cells to the endothelium of the retinal vessels continued until death (in CBA/T6 mice) or resolution (in DBA/2J mice). Adherent monocytes, particularly in PbA-CBA mice, were associated with reduced Hoechst staining of individual endothelial cells and a banking up proximally of both parasitized and nonparasitized blood cells in the small blood vessels, often with accompanying focal leakage of Evans' Blue from the retinal vessels. The occurrence and severity of these early changes in the microcirculation correlated with the subsequent development of cerebral symptoms. Monocyte margination appeared to be the most significant factor associated with the development of cerebral symptoms (73).

Table 3 summarizes some immunohistopathological features

TABLE 3. Brain histopathology of CM in the human disease and the PbA model

Parameter	Human CM	PbA model
Brain hemorrhage	++	++
Plugging of microvessels	++	++
Sequestration of pRBC	+++	+
Knobs on pRBC	++	−
Sequestration of leukocytes	++	+++
Sequestration of platelets	++	++
Necrosis of microvessels	+	+
CAM upregulated	ICAM-1, VCAM-1, E-selectin, TSP, CD36	ICAM-1, VCAM-1
Overexpression of MHC	Class I and II	Class I and II
Upregulation of TNF receptors	p75	p75

of the CM model induced by PbA in CBA/J mice, the details of which have been described elsewhere (31). In this model, cerebrovascular pathological changes are the main features. A sequestration of pRBC, the hallmark of human CM, has been also found, albeit to a lesser extent, in CM-susceptible mice on infection with PbA (42a, 46; P. F. Piguet and G. E. Grau, unpublished data).

Cytokine Interplay Leading to TNF Overproduction

Previous results suggesting that TNF is a key element in the pathogenesis of experimental CM have been reviewed in detail elsewhere (36). These include the observation of high levels of this cytokine is serum at the time of CM, prevention of the neurological syndrome by the in vivo neutralization of the cytokine, and induction of a CM-like syndrome in CM-resistant mice by infusion of the cytokine. More recently, additional confirmation of the pathogenic role of TNF in CM has been provided in experiments with transgenic mice expressing high levels of TNF receptor 1 (TNFR1) (28) and in TNF/LT gene knockout mice (91). Clinical trials with anti-TNF monoclonal antibody (MAb) in humans have led to a significant drop in malarial fever, implying a role of TNF as an endogenous pyrogen (54), but mortality and morbidity were not reduced. Possible reasons for this lack of efficacy include late administration, well after irreversible lesions have occurred, and a carrier effect of the MAb (115).

While TNF production can be triggered directly by the malaria parasite (3), we propose that for CM to occur, there is a need for an amplification loop which involves T-cell activation. TNF release is triggered directly by malaria parasites via both protein kinase C and calmodulin-dependent protein kinase activation, with a regulation that differs from that of lipopolysaccharide (81). The receptor involved in the parasite-induced monocyte/macrophage stimulation has not been characterized. Malaria toxins are important molecules that are responsible for the direct induction by the parasite of TNF secretion by host monocytes (6, 51, 53, 92, 93; D. Kwiatkowski, Reply, Parasitol. Today **11**:463, 1995). Similarly, malaria parasites might induce intercellular cell adhesion molecule 1 (ICAM-1) upregulation directly, in a TNF-independent fashion (21).

The requirement for T lymphocytes in CM pathogenesis has been suggested by observations that athymic nude mice do not develop this neurological syndrome on infection with PbA (25). The respective role of T-cell subsets in the triggering of CM has been analyzed: in the PbA model, anti-CD4 but not anti-CD8 monoclonal antibody significantly reduced serum TNF levels (33) and completely abrogated the occurrence of CM (38), while in the *P. berghei* NK65 model, anti-CD8 but not anti-CD4 antibody protected rats against brain pathology (45).

In experimental CM, the cytokine characteristic of Th1 cells, IFN-γ, plays a significant role. The treatment of PbA-CBA mice with neutralizing anti-IFN-γ MAbs significantly decreased serum TNF levels and prevented the development of cerebral pathology (34). Increased IFN-γ mRNA levels were found in CM-susceptible but not CM-resistant mice during the neurological syndrome. IFN-γ increases the TNF mRNA level (49) and upregulates the TNF receptors on the target cell surface (108). A synergy between IFN-γ and TNF, particularly with respect to the effects on endothelial cells, has also been demonstrated (83).

The IFN-γ production capacity appears to be one of the important parameters associated with the susceptibility to CM: CM-susceptible mice exhibit a preferential expansion of Th1-like clones that is characterized by a marked production of IFN-γ (16). Individuals at risk for severe malaria also produce more IFN-γ, in an antigen-specific manner, than do those who are immune and relatively protected against malaria complications (11). Although these findings may explain why cytokines such as TNF or IFN-γ are overproduced during CM and are sometimes detectable in high concentrations in mice or patients with CM, they cannot explain the phenomenon of tolerance to TNF. The recent observation that TNF differentially regulates the expression of its own receptors in CM-susceptible and CM-resistant brain endothelium (see below) may represent a complementary explanation. The kinetics and site of production of IFN-γ are other important parameters: a recent study indicates that a very early peak of IFN-γ production in the spleen is higher in nonlethal than in lethal murine malaria (17).

Involvement of Cell Adhesion Molecules

Since TNF can induce or upregulate various cell adhesion molecules (CAM) on endothelial cells, the expression of these molecules was analyzed by immunohistochemistry. Brain vessels from mice with CM showed a marked upregulation of ICAM-1 (22, 39) and vascular cell adhesion molecule 1 (VCAM-1) (unpublished data). We attempted to prevent CM by intravenous injection of MAbs directed against LFA-1, Mac-1, ICAM-1, VCAM-1, VLA-4 and P-selectin; only anti-LFA-1 MAb proved to be efficient as described below (see "Assessment of various effector cells in the neurovascular lesion"). The important role of ICAM-1 was confirmed using a SCID mouse model in which *P. falciparum*-infected human RBC adhere to brain ICAM-1 (120), and more recently using ICAM-1-deficient mice (23).

IN VITRO MODELS OF CEREBRAL MALARIA

In human CM, the attachment of RBC infected with mature-stage parasites to endothelial cells lining the postcapillary venules is not restricted to the brain. Microvessels of the heart, lungs, kidneys, small intestine, and liver are the principal sites of sequestration. This sequestration is important for the survival of the parasite but may have severe consequences for the host (32). Sequestered cells that clog the brain capillaries may

reduce blood flow sufficiently that confusion, lethargy, and unarousable coma (i.e, CM) result. The molecular characteristics of the surface proteins, that is, the RBC receptors and the endothelial cell ligands, involved in sequestration have been reviewed (99).

One of the specific features of human RBC on infection by *P. falciparum* is the formation of membrane knobs (12; reviewed in reference 98). Such structures are not found on mouse pRBC. Although pRBC with knobs indeed exhibit high adhesion capacities, the knobs are not essential for cytoadherence because pRBC without knobs were also found to bind efficiently in vitro (113). Thus, cytoadherence occurs irrespective of the presence of knobs, and, conversely, the knobby phenotype does not necessarily lead to a greater ability to cytoadhere (90). In addition, pRBC infected with *P. yoelii* 17XL adhered to mouse brain MVEC, indicating that the absence of knobs on mouse pRBC does not hinder their sequestration in mice with CM (48). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on the surface of pRBC, specifically binds to CD36 and thrombospondin. In addition, *P. falciparum*-infected RBC can express a lymphocyte function antigen 1 (LFA-1)-like molecule that adheres to human umbilical vein endothelial cells (HUVEC) or human brain MVEC (104). Parasites isolated from patients with CM, severe malaria, or uncomplicated malaria exhibit similar adherence properties: there does not seem to be a correlation between cytoadherence and disease severity (30, 88). These parasites exhibit a different capacity for rosette formation, suggesting that rosette formation may be another factor that contributes to the sequestration of pRBC (24); however, this has not been found by other authors (88).

Several adhesion molecules mediate the cytoadherence of pRBC to endothelial cells, including thrombospondin, CD36, ICAM-1, VCAM-1, endothelial cell leukocyte adhesion molecule 1 (ELAM-1, E-selectin), chondroitin sulfate A, and, more recently, P-selectin (43), αVβ3, and platelet-endothelial cell adhesion molecule 1 (CD31) (107). Synergism has been described between CD36 and ICAM-1. Furthermore, under flowing rather than static conditions, pRBC adherence proves to be a multistep process, involving various CAMs (114). This aspect of CM pathogenesis has been reviewed (44).

Numerous cell types have been established to investigate the adhesion molecules for pRBC adherence in vitro. These models include HUVEC, C32 amelanotic melanoma cells, human monocytes, human platelets, U937 myelomonocytic cells (111) and Chinese hamster ovary (CHO) cells transfected with genes coding for CD36 or ICAM-1 (42). Recently, brain MVEC isolated from humans, mice, and monkeys have been used to study the cytoadherence mechanisms (Table 4). Several groups have compared the cytoadherence of pRBC using these models, and their results suggested that CD36 but not ICAM-1 is the principal receptor mediating the cytoadherence of pRBC. This has especially been clear when HB-MVEC and HUVEC were directly compared. HUVEC, with no or only low expression of CD36, display only a low binding capacity for pRBC (102). Interestingly, when comparing the expression of adhesion molecules on brain MVEC between human and murine CM, the main difference is the absence of a known equivalent for CD36 in murine brain MVEC. This may be one of the reasons why the sequestered cells are leukocytes rather than pRBC in experimental CM.

TABLE 4. Some in vitro models for the study of pRBC cytoadherence

System	ICAM-1[a]	CD36[a]	Reference(s)
HUVEC	+	−	112
C32	+	++	5
Monocytes	+	±	5
Platelets	−	+++	75
Stably transfected cells (CD36)	−	+++	42, 77
Stably transfected cells (ICAM-1)	+++	−	42
HD-MVEC	+	+	119
HB-MVEC	+	+	47, 102
HBEC-5I (immortalized)	+	+	121
BB19 (immortalized)			86
HL-MVEC	+	+	71
Monkey brain MVEC	+	+	29
Mouse brain MVEC	+	−	56, 69
Retinal wholemount	+	−	10

[a] Symbols indicate the degree to which this adhesin molecule was present.

pRBC adhere not only to endothelial cells but also to platelets and monocytes, since the receptors which mediate cytoadherence for some pRBC (CD36 and ICAM-1) also exist on these cell types. This process may participate in microvessel plugging in CM.

Retinal Whole-Mount Model

Changes in the cerebral microvasculature, such as breakdown of the blood-brain barrier, petechial hemorrhages, congestion, and edema, are observed in the later stages of murine CM and have been studied in an elegant retinal whole-mount model (10). The retinal vasculature offers a unique opportunity to study rheologic, barrier, and functional properties of the microvasculature within a normal spatial relationship with other tissues and as an intact vascular plexus. A combination of techniques, developed to examine the progressive microvascular changes in murine CM, detected phenomena such as monocyte adherence to endothelial cells, congestion, small haemorrhages, and breakdown of the blood-retina barrier, including details of the location of this leakage, earlier than was possible by studying brain sections. In addition, the covisualization of the blood elements, barrier properties, and vascular endothelial integrity that is possible with retinal whole mounts allowed a detailed analysis of the interaction of different cellular elements in the pathogenesis of CM. Except for the detection of edema, the retinal whole-mount technique offers a more powerful and less time-consuming technique for detecting early microvascular changes in murine CM.

The retinal whole-mount system has also been used to evaluate the pathogenic importance of glial cells in CM (67). Indeed, little attention had been given to the repercussion of microvascular damage on this important cell type during CM. The changes in astrocyte morphology and distribution were compared in three settings: a fatal model, a "resolving" model, and a non-CM model. In the fatal model, retinal astrocytes lost their even distribution from day 3 postinoculation (p.i.) with malaria parasites, progressing to gliosis (day 5 p.i.), well before the onset of cerebral symptoms on day 6 to 7 p.i. At the terminal stage of the disease, there was a loss of astrocyte

processes contacting retinal vessels, often along vessel segments containing adherent monocytes. These features occurred in a mild form in the resolving model and were absent in the non-CM model. Manipulation of these experimental models indicates that astrocytes are involved in the pathogenesis of CM and that the initial changes in astrocyte distribution may be a consequence of the increase in blood-retina barrier permeability; the immune response triggered by the malaria parasite may also be responsible for the loss of astrocyte ensheathment of vessel segments. Moreover, glial cells, particularly microglia and astrocytes, in addition to monocytes, have been shown recently to represent an important source of TNF in CM (68).

Brain MVEC

Alterations in cerebral microvessels have long been recognized to be central in the pathology of CM. The first description of sequestration, i.e., the obstruction of cerebral microvessels by large numbers of mature pRBC, by Marchiafava and Bignami in 1894, has been confirmed by several authors (9, 32). The nonsequestering malaria parasites rarely cause severe illness or death, so that the association between histological and clinical parameters is clear. The causal relationship, however, is not established and remains a subject of debate (9, 14, 32). Descriptions of sequestration content vary. It has been claimed that only mature forms of the parasite would be sequestered, but it has been shown recently that all stages of *P. falciparum* are sequestered in the brain (100) and that immature forms may be particularly important (85a). Also, it has been claimed that only pRBC are present in brain microvessels, but macrophages and monocytes have also been described (18, 76, 78, 85). In a recent study of Malawian children with CM, sequestered pigmented macrophages were significantly more numerous in CM patients than in noncerebral malaria patients (C. D. Mackenzie, G. E. Grau, R. Carr, N. G. Liomba, M. E. Molyneux, and T. E. Taylor, Proc. 48th Meet. Am. Soc. Trop. Med. Hys., abstr. 781, p. 476, 1999).

Given this central role of the cerebral endothelium in CM pathogenesis, MVEC have been isolated from almost any organ or tissue (94). MVEC differ from large-vessel endothelial cells (LVEC) by various morphological and functional variables (35, 57). In addition, endothelial cells of arterial origin are different from those of venous origin (7) and show heterogeneity even within a tissue itself, e.g., the dermis (79). Moreover, MVEC derived from various organs also differ in some characteristics (8). Indeed, MVEC derived from different areas of the microcirculation exhibit differential adhesive properties for granulocytes (55). These data suggest that LVEC may not be adequate for the study of pathological events occurring in microvessels. In view of the organ specificity of endothelial cells, MVEC should be derived from the tissue involved in the diseases one wishes to study (94).

Only studies on human brain MVEC address this issue directly. Some studies have dealt with human brain MVEC (47, 102, 104), and have shown, among other things, that CD36 is a crucial molecule for *P. falciparum*-infected RBC binding. CD36 is indeed one of the differences between LVEC and MVEC. Human dermal also express CD36 but may be different from their brain counterparts (119). Studies using human brain MVEC have allowed the description of potentially new receptors for pRBC (121) and an evaluation of the effect of polyanions on cytoadherence as well as invasion (122). Prudhomme et al. have characterized an immortalized human brain capillary endothelial cell line, named BB19 (86). These cells, on transformation with the E6E7 genes of human papillomavirus, retained their endothelial nature, as shown by phenotypic and functional tests. Interestingly, the BB19 cells bound RBC infected with the FCR-3 and ITO4 strains of *P. falciparum*, making the BB19 cell line a useful system in the analysis of receptor-based cytoadherence and sequestration.

RECENT EXPERIMENTAL RESULTS WITH THE *P. BERGHEI* ANKA MODEL

Assessment of Various Effector Cells in the Neurovascular Lesion: Pathogenic Role of Platelets

A possible role of platelets in CM pathogenesis was adduced from experiments with anti-integrin antibodies in PbA-infected animals. Treatment of infected mice with an antibody against LFA-1 prevented a fatal outcome even when given within minutes before death (22, 39). However, the mechanism of action of anti-LFA-1 MAb remained obscure, since it did not significantly diminish mononuclear cell sequestration. To investigate the mechanism of action of anti-LFA-1 MAb, we searched for other cell types involved in the neurovascular lesion. Given the known accumulation of blood platelets in the microvasculature of mice developing other types of immunopathological reactions (82), we investigated their possible role in experimental CM.

In this in vivo model, four lines of evidence suggest that platelets are critical effectors of the neurovascular injury. First, electron microscopic analysis during CM disclosed platelets in the lumen of brain venules between sequestered monocytes and infected RBC. Platelets were often adherent to damaged endothelial cells, and some appeared fused in the cytoplasm of endothelial cells (40). In contrast, blood mononuclear cells did not show evidence of fusion with brain endothelial cells while in close contact with these cells.

Second, radiolabeled platelet distribution studies indicated that platelets were sequestered in the brain and lung vasculature during CM. Noncerebral malaria was not associated with cerebral sequestration of platelets. Third, in vivo treatment with a MAb to LFA-1 (which is expressed on platelets) selectively abrogated the cerebral sequestration of platelets, and this correlated with prevention of CM. Fourth, the role of platelets in the development of neurovascular lesion of CM was more directly assessed by a depletion experiment. Malaria-infected animals rendered thrombocytopenic were significantly protected against CM, further indicating that platelets are central to the pathogenesis of CM. Thus, a CD11a-dependent interaction between platelets and endothelial cells appears pivotal to microvascular damage. These data suggest a novel mechanism of action for anti-LFA-1 MAb in vivo and illustrate an unexpected role for platelets, in addition to monocytes, in vascular pathology.

Thus, significant platelet sequestration in the brain occurs during CM and is abrogated by anti-LFA-1 MAb treatment. Surface LFA-1 might confer on platelets the ability to bind to

ICAM-1 present on endothelial cells and to fuse with them. This markedly increased fusion, occurring at a late stage in CM, would then lead to irreversible endothelial cell damage and ensuing hemorrhages. This could also explain why delayed administration of anti-LFA-1 MAb can still be protective, since it would interfere with these late-occurring effector interactions. The fusion of platelets of EC has been described in vitro and in vivo as a physiological process, i.e., capable of exerting a trophic role for endothelial cells. In severe malaria, the fusion rate might be accelerated because of the increased amounts of ICAM-1 and would lead to endothelial damage.

Our present results suggest that the real effector of vascular damage might not be the most abundant cells (monocytes) in brain microvessels, since these cells are still sequestered after anti-LFA-1 MAb treatment. Also, there is no direct evidence that pRBC inflict venular injury. It therefore seems possible that in mouse CM, the critical effector of venular damage is, in fact, the less obvious platelets. Platelets are generally considered to be involved in hemostasis, but there is also evidence of their toxicity, since they are capable of killing various cells including malaria parasites (84). Results obtained with the mouse CM model demonstrate that platelets can also act as effector of immunopathological reactions, notably by damaging endothelial cells, thus producing hemorrhages, the lesion they are commonly recognized to arrest. Recognition of this effector mechanism might have widespread application in other types of TNF-induced hemorrhagic necrosis and could represent an alternative mechanism of action of anti-LFA-1 MAb in vivo.

Results obtained in vivo with the mouse CM model suggested that platelets may have a detrimental effect on endothelial functions. Since TNF and IFN-γ are important mediators in experimental CM, the relation between TNF/IFN-γ and platelet adhesion/fusion was further investigated in vitro using cocultures of brain MVEC and platelets (57).

The mechanisms of the fusion phenomenon were evaluated, with particular attention to the role of the adhesion molecules involved and to its functional consequences. TNF was found to induce the adhesion of radiolabeled platelets to MVEC in a dose-dependent manner. This effect was amplified by IFN-γ. Conversely, MAbs to ICAM-1 or to one of its ligands, LFA-1, abrogated the adhesion of platelets induced by these cytokines. Electron microscopic examination showed that platelets adhered and fused to endothelial cells. A fluorescent dye was to found to be transferred from labeled platelets into endothelial cell cytoplasm, provided that these endothelial cells were prestimulated with TNF. In addition, platelet surface markers, such as LFA-1 or the platelet antigen recognized by our antiplatelet MAb, CV5-H7, were transferred to endothelial cell membranes. The addition of platelets to TNF-activated endothelial monolayers caused enhanced cytotoxicity, as shown by ^{51}Cr release assays. Finally, binding studies indicated that adhesion and fusion of platelets to MVEC significantly increased their adhesiveness for blood leukocytes. These data indicate that fusion of platelets into MVEC, (i) is synergistically induced by TNF and IFN-γ, (ii) is dependent on the β_2-integrin LFA-1 expressed on the platelet surface, (iii) participates is the platelet-mediated enhancement of endothelial injury, and (iv) critically modulates the cytoadherence capacity of MVEC for leukocytes, thereby representing an important mechanism in the modulation of TNF effects in microvascular pathology (Fig. 1).

Pathogenic Role of TNFR2 (p75) in CM Pathology

After studying the role of effector cells in TNF-induced endothelial alterations, we analyzed the respective roles of the two TNF receptors, TNFR1 (p55) and TNFR2 (p75) (59). The immunohistochemical examination of brain sections from PbA-infected CM-susceptible mice revealed a significant upregulation of ICAM-1 and TNFR2, but not TNFR1, in capillaries and venules. To evaluate the respective role of each receptor in CM pathogenesis, we subsequently investigated the susceptibility of TNFR1- or TNFR2-deficient mice (*Tnfr1*^{0} and *Tnfr2*^{0}, respectively) to the neurological syndrome. Surprisingly, in contrast to most other infectious diseases in which TNF is involved that indicate a role for TNFR1 (26, 80, 89, 103, 116), protection from CM was found in *Tnfr2*^{0} mice but not in *Tnfr1*^{0} mice (60). To explain the resistance of the *Tnfr2*^{0} mice, we first investigated whether there were differences in the levels of critical mediators of CM, such as TNF and IFN-γ, in these mice. This was not the case: no significant differences were found in the levels of TNF, IFN-γ, soluble TNFR1 (sTNFR1), and sTNFR2 in blood, except for the absence of sTNFRs in the corresponding knockout mice. Second, we addressed the question of a different sensitivity to TNF in Tnfr2^0 mice. Since brain MVEC isolated from the *Tnfr1*^{0} and *Tnfr2*^{0} mice both showed reduced sensitivity to the TNF-induced cytotoxicity compared to that shown by cells isolated from the wild-type mice, this result cannot explain the specific protection of the *Tnfr2*^{0} mice. We then examined the possibility that ICAM-1 could be differentially modulated in mice lacking one of the two TNFR. Indeed, in experimental CM, it has been shown that ICAM-1 upregulation in endothelial cells contributes to the subsequent adherence of leukocytes. Interestingly, the brain sections isolated from the CM-resistant PbA-infected *Tnfr2*^{0} mice did not show the leukocyte sequestration and ICAM-1 upregulation that occurred in the CM-susceptible wild type or *Tnfr1*^{0} mice.

Since the soluble TNF-mediated ICAM-1 upregulation on brain MVEC in vitro is exclusively mediated by the TNFR1, we then investigated whether membrane-bound TNF, which has recently been shown to preferentially interact with TNFR2, can explain the apparent discrepancy between the in vitro and the in vivo results (60). In experiments with MVEC isolated from wild-type, *Tnfr1*^{0}, or *Tnfr2*^{0} mice, we showed that soluble TNF requires the presence of both TNF receptors whereas membrane-bound TNF needs only TNFR2 for TNF-mediated ICAM-1 upregulation in brain MVEC. It was seen that in MVEC lacking TNFR2 only, neither membrane-bound nor soluble TNF can upregulate ICAM-1 in vitro. As controls, *Tnfr2*^{0} MVEC were still capable of upregulating ICAM-1 on stimulation by a stimulus different from TNF namely, CD40L (kindly provided by J. Y. Bonnefoy, Glaxo-Wellcome, Geneva Switzerland). Conversely, as another control, TNF was still able to increase the expression of E-selectin on *Tnfr2*^{0} MVEC. In conclusion, these results indicate that the interaction between membrane TNF and TNFR2 is crucial to the development of the neurological syndrome seen in severe malaria (59). More recently, TNFR2 was shown to be important in endo-

thelial cell apoptosis in the absence of sensitizing agents, i.e., under pathophysiologically relevant conditions (58).

Role of Endothelial Cells in Genetic Susceptibility to CM: Comparison of Brain MVEC Derived from Susceptible and Resistant Mice

The reasons why only a small number of individuals infected with *P. falciparum* develop cerebral malaria remain obscure. There is evidence that the nature of the malaria parasite as well as the host genetic background may partially explain this phenomenon (3, 66). In experimental models of CM, different mouse strains, for unknown reasons, also exhibit different sensitivities to CM (25, 38, 63) (Table 2).

It has been shown that overproduction of TNF contributes to the pathology of CM (33) and is related to the severity of the cerebral syndrome (41). In other studies, some patients with high levels of TNF in blood did not develop CM while some patients with low levels of TNF did (97), suggesting differential hosts sensitivity to TNF. Since brain MVEC are an important target for TNF action in experimental CM, we investigated whether these cells derived respectively from CM-susceptible and CM-resistant mice exhibit a different responsiveness to TNF.

Brain MVEC purified from CM-susceptible (CM-S) CBA/J mice and CM-resistant (CM-R) BALB/c mice indeed exhibit different sensitivities to TNF in terms of cytokine production, adhesion molecule expression and TNF receptor expression (56). CM-S brain MVEC displayed a higher capacity to produce interleukin-6 and to upregulate ICAM-1 and VCAM-1 in response to TNF than CM-R brain MVEC. In contrast, no difference was found in the induction of E-selectin after TNF challenge. CM-S brain MVEC were also significantly more sensitive to TNF-induced lysis. This differential reactivity to TNF was further substantiated by comparing TNFR expression on CM-S and CM-R brain MVEC. Although the constitutive expression of TNFRs was comparable on cells from the two origins, TNF induced an upregulation of both p55 and p75 TNFRs in CM-S but not in CM-R brain MVEC. A similar regulation was found at the level of TNFR mRNA but not at the level of receptor shedding. Although a protein kinase C inhibitor blocked the response to TNF in brain MVEC of both CM-S and CM-R mice, an inhibitor of protein kinase A selectively abolished the response to TNF in CM-R but not CM-S brain MVEC, suggesting a differential protein kinase involvement in TNF-induced activation of CM-S and CM-R brain MVEC. These results indicate that brain MVEC purified from CM-S and CM-R mice exhibit distinctive sensitivities to TNF. This difference may be partly due to a differential regulation of TNF receptors and via distinct protein kinase pathways (56).

Since CM also involves IFN-γ that is known to modulate major histocompatibility complex (MHC) molecule expression, we also evaluated whether the genetic susceptibility to CM is related to the constitutive or IFN-γ-induced expression of MHC molecules on brain microvessels (69). By flow cytometry, we found that less than 5% of CM-S brain MVEC constitutively expressed MHC class I molecules, in contrast to up to 90% of CM-R brain MVEC. On stimulation with IFN-γ the percentage of positive cells for MHC class I molecules in CM-S brain MVEC became comparable to that in CM-R brain MVEC, but a higher fluorescence intensity existed on CM-S brain MVEC than on CM-R brain MVEC. MHC class II molecules were not constitutively expressed on brain MVEC from either strain. IFN-γ-induced expression of MHC class II (I-A, I-E) molecules was significantly higher in CM-S than CM-R brain MVEC in terms of both the percentage of positive cells and the fluorescence intensity. In addition, an obvious protection from experimental CM was found in B6-Aa^0/Aa^0 mutant mice, which lack cell surface expression of MHC class II molecules, indicating the importance of MHC class II molecules in CM pathogenesis. These data demonstrate that differential inducibility of MHC class II expression on brain MVEC is correlated with a genetic susceptibility to CM (69).

PERSPECTIVES

Overall Relevance of Models: Human versus Murine Pathology

Experimental CM cannot reproduce exactly the brain pathology of this complication in humans, but there is a growing number of similarities between several models and the human disease (Table 3). In particular, in the PbA model, the presence of mononuclear cells in brain venules finds a parallel in pediatric CM, since there is a substantial accumulation of monocytes and pigmented macrophages (62) in brain vessels. Furthermore, the number of these leukocytes is significantly greater in CM than in noncerebral malaria patients. Another parameter in common between human and murine CM is the upregulation of TNFR2 on brain microvessels (62) (Table 3).

The accumulation of platelets in the brain vessels of these children is also significantly more important in CM than in noncerebral malaria patients (G. E. Grau, C. D. Mackenzie, R. Carr, M. Redard, G. P. Pizzolato, P. Moulin, C. Cataldo, N. G. Liomba, M. E. Molyneux, and T. E. Taylor, Proc. 48th Meet. Am. Soc. Trop. Med. Hyg., abstr. 780, p. 475, 1999), this represents another new factor in common with the PbA model. Conversely, as mentioned above, there is also pRBC accumulation in the brain of PbA-infected mice, although this is less marked than in other murine models.

Platelet-Endothelial Interactions in Microvascular Pathology Beyond CM

The effects of anti-LFA-1 MAb on CM and on platelet accumulation in brain vessels may offer insight into the mechanism of action of this antibody in vivo. Besides CM, in vivo treatment of mice with anti-integrin MAbs confers protection against diverse types of experimental diseases, such as diabetes, transplant rejection, encephalomyelitis, and pulmonary fibrosis (reviewed in reference 36). These data imply that apart from their beneficial role in hemostasis, platelets should also be viewed as pathogenic effectors in vascular lesions. More recently, in vitro experiments with human cells have indicated a role for LFA-1 in platelet-endothelium interactions, substantiated the fusion phenomenon, and confirmed that platelets can potentiate the TNF-induced endothelial killing (L. Camoin et al., submitted for publication).

A pathogenic role for platelets is also suspected in disorders other than CM: gram-negative bacterial septic shock and acute respiratory distress syndrome, vasculitides (e.g., systemic lupus

erythematosus), pulmonary fibrosis, tumor metastasis, transplant rejection, and stroke, brain hypoxia, and related conditions (reviewed in reference 64). Indeed, platelets have been detected during rejection episodes (70); particularly bound to ischemic kidney allografts (50). While both condition seem to involve platelets, ischemia-reperfusion (IR) may differ from CM in some aspects. In murine CM, the adhesion of platelets can be regarded as a particular type of thrombosis. Indeed, in this model, platelet depletion does not correlate with fibrinogen consumption (96), while fibrinogen deposition is a prerequisite for platelet adhesion in IR injury (65). Interestingly, in human CM, fibrinogen is deposited inside the vessel lumen, i.e., among sequestered pRBC, leukocytes, and platelets, rather than along the endothelial lining, the pattern seen in IR (Grau et al., Proc. 48th Meet. ASTMH; Mackenzie et al., Proc. 48th Meet. ASTMH). This pattern is consistent with the data of murine CM and is compatible with a less important role of fibrinogen in CM.

Recently, additional in vivo evidence of a pathogenic role of platelets has been obtained in experiments with uPAR (CD87) KO mice (P. F. Piguet et al., Infect. Immun, in press). These mice are resistant to CM when infected with Quantitative immunohistochemistry for GPIIb-IIIa revealed that the platelet sequestration occurring in wild-type mice which develop neurological signs was prevented in uPAR KO mice. The uPAR deficiency did not disturb the immune response leading to TNF overproduction, supporting the hypothesis that platelets are acting as effectors of the neurovascular lesion.

The mechanisms by which platelets act as effectors are potentially numerous: a pathogenic role of platelets can be envisaged at several levels. Platelets can alter endothelial functions in numerous ways directly and/or indirectly via a modulation of leukocyte functions and even via effects on normal RBC and pRBC (reviewed in reference 64).

The relative importance of these numerous effects of platelets in CM pathogenesis remains to be established. Based on reports describing only the pRBC sequestration in human CM (2, 95), attention has focused on developing a model of pRBC binding. The presence of leukocytes and platelets in human CM lesions, however, prompts the need for studying three-party and even four-party-interaction models in more depth. A better understanding of these complex interactions leading to vascular injury can help us improve the outcome of this disease.

REFERENCES

1. **Aikawa, M., A. E. Brown, C. D. Smith, T. Tegoshi, R. J. Howard, T. H. Hasler, Y. Ito, W. E. Collins, and H. K. Webster.** 1992. *Plasmodium-coatneyi*-infected rhesus monkeys—a primate model for human cerebral malaria. Mem. Inst. Oswaldo Cruz **87**:443–447.
2. **Aikawa, M., E. Pongponratn, T. Tegoshi, K. I. Nakamura, T. Nagatake, A. Cochrane, and L. S. Ozaki.** 1992. A study on the pathogenesis of human cerebral malaria and cerebral babesiosis. Mem. Inst. Oswaldo Cruz **87**: 297–301.
3. **Allan, R. J., A. Rowe, and D. Kwiatkowski.** 1993. *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. Infect. Immun. **61**: 4772–4776.
4. **Bafort, J. M., W. H. Pryor, and J. M. Ramsey.** 1980. Immunization of rats against malaria: a new model. J. Parasitol. **66**:337–338.
5. **Barnwell, J. W., C. F. Ockenhouse, and D. M. Knowles.** 1985. Monoclonal antibody OKM5 inhibits in vitro binding of *Plasmodium falciparum* infected erythrocytes to monocytes, endothelial, and C32 melanoma cells. J. Immunol. **135**:3494–3497.
6. **Bate, C. A. W., and D. P. Kwiatkowski.** 1994. Stimulators of tumour necrosis factor production released by damaged erythrocytes. Immunology **83**:256–261.
7. **Beekhuizen, H., and R. Vanfurth.** 1994. Growth characteristics of cultured human macrovascular venous and arterial and microvascular endothelial cells. J. Vasc. Res. **31**:230–239.
8. **Belloni, P. N., D. H. Carney, and G. L. Nicolson.** 1992. Organ-derived microvessel endothelial cells exhibit differential responsiveness to thrombin and other growth factors. Microvasc. Res. **43**:20–45.
9. **Berendt, A. R., G. D. H. Turner, and C. I. Newbold.** 1994. Cerebral malaria: the sequestration hypothesis. Parasitol. Today **10**:412–414.
10. **Chan-Ling, T., A. L. Neill, and N. H. Hunt.** 1992. Early microvascular changes in murine cerebral malaria detected in retinal wholemounts. Am. J. Pathol. **140**:1121–1130.
11. **Chizzolini, C., G. E. Grau, A. Geinoz, and D. Schrijvers.** 1990. Lymphocyte-T interferon-gamma production induced by *Plasmodium falciparum* antigen is high in recently infected non-immune and low in immune subjects. Clin. Exp. Immunol. **79**:95–99.
12. **Clark, I. A., G. Chaudhri, and W. B. Cowden.** 1989. Roles of tumour necrosis factor in the illness and pathology of malaria. Trans. R. Soc. Trop. Med. Hyg. **83**:436–440.
13. **Clark, I. A., J. D. Macmicking, K. M. Gray, K. A. Rockett, and W. B. Cowden.** 1992. Malaria mimicry with tumor necrosis factor—contrasts between species of murine malaria and *Plasmodium falciparum*. Am. J. Pathol. **140**:325–336.
14. **Clark, I. A., and K. A. Rockett.** 1994. The cytokine theory of human cerebral malaria. Parasitol. Today **10**:410–412.
15. **Curfs, J. H. A. J., C. C. Hermsen, P. Kremsner, S. Neifer, J. H. E. T. Meuwissen, N. Vanrooyen, and W. M. C. Eling.** 1993. Tumour necrosis factor-alpha and macrophages in *Plasmodium-berghei*-induced cerebral malaria. Parasitology **107**:125–134.
16. **de Kossodo, S., and G. E. Grau.** 1993. Profiles of cytokine production in relation with susceptibility to experimental cerebral malaria. J. Immunol. **151**:4811–4820.
17. **de Souza, J. B., K. H. Williamson, T. Otani, and J. H. Playfair.** 1997. Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. Infect. Immun. **65**:1593–1598.
18. **Elamin, A. M.** 1981. Cerebral malaria in adult Zambian Africans. East Afr. Med. J. **58**:124–129.
19. **Eling, W. M.** 1980. *Plasmodium berghei*: premunition, sterile immunity, and loss of immunity in mice. Exp. Parasitol. **49**:89–96.
20. **Eling, W. M.** 1982. Chronic, patent *Plasmodium berghei* malaria in splenectomized mice. Infect. Immun. **35**:880–886.
21. **Esslinger, C. W., S. Picot, and P. Ambroisethomas.** 1994. Intra-erythrocytic *Plasmodium falciparum* induces up-regulation of inter-cellular adhesion molecule-1 on human endothelial cells in vitro. Scand. J. Immunol. **39**: 229–232.
22. **Falanga, P. B., and E. C. Butcher.** 1991. Late treatment with anti-LFA-1 (CD11a) antibody prevents cerebral malaria in a mouse model. Eur. J. Immunol. **21**:2259–2263.
23. **Favre, N., C. DaLaperousaz, B. Ryffel, N. A. Weiss, B. A. Imhof, W. Rudin, R. Lucas, and P. F. Piguet.** 1999. Role of ICAM-1 (CD54) in the development of murine cerebral malaria. Microbes. Infect. **1**:961–968.
24. **Fernandez, V., C. J. Treutiger, G. B. Nash, and M. Wahlgren.** 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect. Immun. **66**:2969–2975.
25. **Finley, R. W., L. J. Mackey, and P. H. Lambert.** 1982. Virulent *P. berghei* malaria: prolonged survival and decreased cerebral pathology in T-cell-deficient nude mice. J. Immunol. **129**:2213–2218.
26. **Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom.** 1995. Tumor necrosis factor-alpha is required in the protective immune response against mycobacterium tuberculosis in mice. Immunity **2**:561–572.
27. **Fujioka, H., P. Millet, Y. Maeno, S. Nakazawa, Y. Ito, R. J. Howard, W. E. Collins, and M. Aikawa.** 1994. A nonhuman primate model for human cerebral malaria: rhesus monkeys experimentally infected with *Plasmodium fragile*. Exp. Parasitol. **78**:371–376.
28. **Garcia, I., K. Araki, Y. Miyazaki, M. Araki, P. Juillard, G. E. Grau, G. Milon, Y. Belkaid, C. Montini, W. Lesslauer, and P. Vassalli.** 1995. Transgenic mice expressing soluble TNF-R1-IgG3 molecules are protected from lethal septic shock and cerebral malaria, and are highly sensitive to *Listeria monocytogenes* and *Leishmania major* infections. Eur. J. Immunol. **25**:2401–2407.
29. **Gay, F., C. Robert, B. Pouvelle, S. Peyrol, A. Scherf, and J. Gysin.** 1995. Isolation and characterization of brain microvascular endothelial cells from saimiri monkeys—an in vitro model for sequestration of *Plasmodium falciparum*-infected erythrocytes. J. Immunol. Methods **184**:15–28.
30. **Goldring, J. D., M. E. Molyneux, T. Taylor, J. Wirima, and M. Hommel.** 1992. *Plasmodium-falciparum*—diversity of isolates from Malawi in their cytoadherence to melanoma cells and monocytes in vitro. Br. J. Haematol **81**:413–418.
31. **Grau, G. E., and S. de Kossodo.** 1993. Role of cytokines and adhesion molecules in malaria immunopathology. Stem Cells **11**:41–48.

32. **Grau, G. E., and S. de Kossodo.** 1994. Cerebral malaria: Mediators, mechanical obstruction or more? Parasitol. Today **10**:408–409.
33. **Grau, G. E., L. F. Fajardo, P. F. Piguet, B. Allet, P. H. Lambert, and P. Vassalli.** 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science **237**:1210–1212.
34. **Grau, G. E., H. Heremans, P. F. Piguet, P. Pointaire, P. H. Lambert, A. Billiau, and P. Vassalli.** 1989. Monoclonal antibody against interferon-gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. Proc. Natl. Acad. Sci. USA **86**:5572–5574.
35. **Grau, G. E., and J. Lou.** 1993. TNF in vascular pathology: importance of platelet-endothelium interactions. Res. Immunol. **144**:355–363.
36. **Grau, G. E., and J. N. Lou.** 1994. TNF-induced microvascular pathology in the Th1-mediated lesions of mouse cerebral malaria—possible new mechanisms, p. 265–299. *In* S. Romagnani, G. DelPrete, and A. K. Abbas (ed.), Cytokines: basic principles and clinical applications Ares-Serono Symposia, Rome, Italy.
37. **Reference deleted.**
38. **Grau, G. E., P. F. Piguet, H. D. Engers, J. A. Louis, P. Vassalli, and P. H. Lambert.** 1986. L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J. Immunol. **137**:2348–2354.
39. **Grau, G. E., P. Pointaire, P. F. Piguet, C. Vesin, H. Rosen, I. Stamenkovic, F. Takei, and P. Vassalli.** 1991. Late administration of monoclonal antibody to leukocyte function-antigen 1 abrogates incipient murine cerebral malaria. Eur. J. Immunol. **21**:2265–2267.
40. **Grau, G. E., F. Tacchini-Cottier, C. Vesin, G. Milon, J. Lou, P. F. Piguet, and P. Juillard.** 1993. TNF-induced microvascular pathology: active role for platelets and importance of the LFA-1/ICAM-1 interaction. Eur. Cytokine Netw. **4**:415–419.
41. **Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P. H. Lambert.** 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. **320**:1586–1591.
42. **Hasler, T., G. R. Albrecht, M. R. Vanschravendijk, J. C. Aguiar, K. E. Morehead, B. L. Pasloske, C. Ma, J. W. Barnwell, B. Greenwood, and R. J. Howard.** 1993. An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am. J. Trop. Med. Hyg. **48**: 332–347.

42a. **Hearn, J., N. Rayment, D.N. Landon, D.R. Katz, and J.B. Desouza.** 2000. Immunopathology of cerebral malaria: morphological evidence of parasite sequestration in murine brain microvasculature. *Infect. Immun.* **68**:5364–5376.

43. **Ho, M., T. Schollaardt, X. F. Niu, S. Looareesuwan, K. D. Patel, and P. Kubes.** 1998. Characterization of *Plasmodium falciparum*-infected erythrocyte and P- selectin interaction under flow conditions. Blood **91**:4803–4809.
44. **Ho, M., and N. J. White.** 1999. Molecular mechanisms of cytoadherence in malaria. Am. J. Physiol. **45**:C1231–C1242.
45. **Imai, Y., and T. Kamiyama.** 1994. T lymphocyte-dependent development of cerebral symptoms in Wm/MS rats infected with *Plasmodium berghei*. Ann. Trop. Med. Parasitol. **88**:83–85,
46. **Jennings, V. M., J. K. Actor, A. A. Lal, and R. L. Hunter.** 1997. Cytokine profile suggesting that murine cerebral malaria is an encephalitis. Infect. Immun. **65**:4883–4887.
47. **Johnson, K. J., R. A. Swerlick, K. K. Grady, P. Millet, and T. M. Wick.** 1993. Cytoadherence of *Plasmodium falciparum*-infected erythrocytes to microvascular endothelium is regulated by cytokines and phorbol ester. J. Infect. Dis. **167**:698–703.
48. **Kaul, D. K., R. L. Nagel, J. F. Llena, and H. L. Shear.** 1994. Cerebral malaria in mice: demonstration of cytoadherence of infected red blood cells and microrheologic correlates. Am. J. Trop. Med. Hyg. **50**:512–521.
49. **Kindler, V., A. P. Sappino, G. E. Grau, P. F. Piguet, and P. Vassalli.** 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell **56**:731–740.
50. **Koo, D. D., K. I. Welsh, J. A. Roake, P. J. Morris, and S. V. Fuggle.** 1998. Ishemia/reperfusion injury in human kidney transplantation: an immunohistochemical analysis of changes after reperfusion. Am. J. Pathol. **153**: 557–566.
51. **Kwiatkowski, D.** 1993. TNF-inducing malaria toxin—a sheep in wolf's clothing. Ann. Trop. Med. Parasitol. **87**:613–616.
52. **Reference deleted.**
53. **Kwiatkowski, D.** 1995. Malarial toxins and the regulation of parasite density. Parasitol. Today **11**:206–212.
54. **Kwiatkowski, D., M. E. Molyneux, S. Stephens, N. Curtis, N. Klein, P. Pointaire, M. Smit, R. Allan, P. Holloway, D. R. Brewster, G. E. Grau, and B. M. Greenwood.** 1993. Response to monoclonal anti-TNF antibody in children with cerebral malaria. Q. J. Med. **86**:91–98.
55. **Ley, K., P. Gaehtgens, and K. Spanelborowski.** 1992. Differential adhesion of granulocytes to five distinct phenotypes of cultured microvascular endothelial cells. Microvasc. Res. **43**:119–133.
56. **Lou, J. N., Y. Gasche, L. Zheng, B. Critico, C. Monsohinard, P. Juillard, P. Morel, W. A. Buurman, and G. E. Grau.** 1998. Differential reactivity of brain microvascular endothelial cells to TNF reflects the genetic susceptibility to cerebral malaria. Eur. J. Immunol. **28**:3989–4000.
57. **Lou, J., Y.R.A. Donati, P. Juillard, C. Giroud, C. Vesin, N. Mili, and G.E. Grau.** 1997. Platelets play an important role in TNF-induced microvascular endothelial cell pathology. Am. J. Pathol. **151**:1397–1405.
58. **Lucas, R., I. Garcia, Y. R. A. Donati, M. Hribar, S. Mandriota, C. Giroud, W. A. Buurman, L. Fransen, P. M. Suter, G. Nunez, M. S. Pepper, and G. E. Grau.** 1998. Both TNF receptors are required for direct TNF-mediated cytotoxicity in microvascular endothelial cells. Eur. J. Immunol. **28**:3577–3586.
59. **Lucas, R., P. Juillard, E. Decoster, M. Redard, D. Burger, Y. Donati, C. Giroud, C. Monsohinard, T. DeKesel, W. A. Buurman, M. W. Moore, J. M. Dayer, W. Fiers, H. Bluethmann, and G. E. Grau.** 1997. Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. Eur. J. Immunol. **27**:1719–1725.
60. **Lucas, R., J. N. Lou, P. Juillard, M. Moore, H. Bluethmann, and G. E. Grau.** 1997. Respective role of TNF receptors in the development of experimental cerebral malaria. J. Neuroimmunol. **72**:143–148.
61. **Luhan, M.** 1996. Malaria. WHO Press Office fact sheet 94, P. 1–3. World Health Organization, Geneva, Switzerland.
62. **Reference deleted.**
63. **Mackey, L. J., A. Hochmann, C. H. June, C. E. Contreras, and P. H. Lambert.** 1980. Immunopathological aspects of *Plasmodium berghei* infection in five strains of mice. II. Immunopathology of cerebral and other tissue lesions during the infection. Clin. Exp. Immunol. **42**:412–420.
64. **Männel, D. N., and G. E. Grau.** 1997. Role of platelet adhesion in homeostasis and immunopathology. J. Clin. Pathol.- Mol. Pathol. **50**:175–185.
65. **Massberg, S., G. Enders, F. C. D. Matos, L. I. D. Tomic, R. Leiderer, S. Eisenmenger, K. Messmer, and F. Krombach.** 1999. Fibrinogen deposition at the post-ischemic vessel wall promotes platelet adhesion during ischemia-reperfusion in vivo. Blood **94**:3829–3838.
66. **McGuire, W., A. V. S. Hill, C. E. M. Allsopp, B. M. Greenwood, and D. Kwiatkowski.** 1994. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature **371**:508–511.
67. **Medana, I. M., T. Chanling, and N. H. Hunt.** 1996. Redistribution and degeneration of retinal astrocytes in experimental murine cerebral malaria: Relationship to disruption of the blood-retinal barrier. Glia **16**:51–64.
68. **Medana, I. M., N. H. Hunt, and G. Chaudhri.** 1997. Tumor necrosis factor-alpha expression in the brain during fatal murine cerebral malaria: Evidence for production by microglia and astrocytes. Am. J. Pathol. **150**: 1473–1486.
69. **Monsohinard, C., J. N. Lou, C. Behr, P. Juillard, and G. E. Grau.** 1997. Expression of major histocompatibility complex antigens on mouse brain microvascular endothelial cells in relation to susceptibility to cerebral malaria. Immunology **92**:53–59.
70. **Mowbray, J.** 1969. Platelet thrombi in rejection episodes. Proc. R. Soc. Med. **62**:597–600.
71. **Muanza, K., F. Gay, C. Behr, and A. Scherf.** 1996. Primary culture of human lung microvessel endothelial cells: an useful in vitro model for studying *Plasmodium falciparum*-infected erythrocyte cytoadherence. Res. Immunol. **147**:149–163.
72. **Najera, J. A., and J. Hempel.** 1996. The burden of malaria. WHO Press Office publication CTD/MAL/96.10, P. 1–58. World Health Organization, Geneva, Switzerland.
73. **Neill, A. L., T. Chanling, and N. H. Hunt.** 1993. Comparisons between microvascular changes in cerebral and non-cerebral malaria in mice, using the retinal whole-mount technique. Parasitology **107**:477–487.
74. **Neill, A. L., and N. H. Hunt.** 1992. Pathology of fatal and resolving *Plasmodium berghei* cerebral malaria in mice. Parasitology **105**:165–175.
75. **Ockenhouse, C. F., N. N. Tandon, C. Magowan, G. A. Jamieson, and J. D. Chulay.** 1989. Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor. Science **243**:1469–1471.
76. **Oo, M. M., M. Aikawa, T. Than, T. M. Aye, P. T. Myint, I. Igarashi, and W. C. Schoene.** 1987. Human cerebral malaria: a pathological study. J. Neuropathol. Exp. Neurol. **46**:223–231.
77. **Oquendo, P., E. Hundt, J. Lawler, and B. Seed.** 1989. CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes. Cell **58**:95–101.
78. **Patnaik, J. K., B. S. Das, S. K. Mishra, S. Mohanty, S. K. Satpathy, and D. Mohanty.** 1994. Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Am. J. Trop. Med. Hyg. **51**:642–647.
79. **Petzelbauer, P., J. R. Bender, J. Wilson, and J. S. Pober.** 1993. Heterogeneity of dermal microvascular endothelial cell antigen expression and cytokine responsiveness *in situ* and in cell culture. J. Immunol. **151**:5062–5072.
80. **Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak.** 1993. Mice deficient for the 55-kDa tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *Listeria monocytogenes* infection. Cell **73**:457–467.
81. **Picot, S., I. Sheick, A. Sylvi, A. Donadille, and P. Ambroise-Thomas.** 1994. Signal transduction pathways involved in tumour necrosis factor secretion

by *Plasmodium falciparum*-stimulated human monocytes. Immunology **83:** 70–74.

82. **Piguet, P. F., H. Rosen, C. Vesin, and G. E. Grau.** 1993. Effective treatment of the pulmonary fibrosis elicited in mice by bleomycin or silica with anti-CD11 antibodies. Am. Rev. Respir. Dis. **147:**435–441.

82a. **Piquet, P. F., C. DaLaperrousaz, C. Vesin, F. Tacchinicottier, G. Senaldi, and G. E. Grau.** 2000. Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. Infect. Immun. **68:**3822–3829.

83. **Pober, J. S., M. A. Gimbrone, L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer.** 1986. Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor, and immune interferon. J. Immunol. **137:**1893–1896.

84. **Polack, B., F. Peyron, L. Kolodie, P. Ambroisethomas, and F. Santoro.** 1990. Platelet receptors involved in *Plasmodium falciparum* growth inhibition. Thromb. Res. **59:**663–667.

85. **Porta, J., A. Carota, G. P. Pizzolato, E. Wildi, M. C. Widmer, C. Margairaz, and G. E. Grau.** 1993. Immunopathological changes in human cerebral malaria. Clin. Neuropathol. **12:**142–146.

85a. **Pouville, B., P. A. Buffet, C. Lepolard, A. Scherf, and J. Gysin.** 2000. Cytoadhesin of *Plasmodium falciparum* ring-stage-infected erythrocytes. Nat. Med. **6:**1264–1268.

86. **Prudhomme, J. G., I. W. Sherman, K. M. Land, A. V. Moses, S. Stenglein, and J. A. Nelson.** 1996. Studies of *Plasmodium falciparum* cytoadherence using immortalized human brain capillary endothelial cells. Int. J. Parasitol. **26:**647–655.

87. **Rest, J. R.** 1983. Pathogenesis of cerebral malaria in golden hamsters and inbred mice. Contrib. Microbiol. Immunol. **7:**139–146.

88. **Rogerson, S. J., R. Tembenu, C. Dobano, S. Plitt, T. E. Taylor, and M. E. Molyneux.** 1999. Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria. Am. J. Trop. Med. Hyg. **61:**467–472.

89. **Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann.** 1993. Mice lacking the tumour necrosis factor receptor-1 are resistant to TNF-Mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature **364:**798–802.

90. **Ruangjirachuporn, W., B. A. Afzelius, S. Paulie, M. Wahlgren, K. Berzins, and P. Perlmann.** 1991. Cytoadherence of knobby and knobless *Plasmodium-falciparum*-infected erythrocytes. Parasitology **102:**325–334.

91. **Rudin, W., H. P. Eugster, G. Bordmann, J. Bonato, M. Muller, M. Yamage, and B. Ryffel.** 1997. Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. Am. J. Pathol. **150:**257–266.

92. **Schofield, L.** 1997. Malaria toxins revisited. Parasitol. Today **13:**275–276.

93. **Schofield, L., and F. Hackett.** 1993. Signal transduction in host cells by a glycosyl phosphatidyl inositol toxin of malaria parasites. J. Exp. Med. **177:** 145–153.

94. **Scott, P. A. E., and R. Bicknell.** 1993. The isolation and culture of microvascular endothelium. J. Cell Sci. **105:**269–273.

95. **Sein, K. K., Y. Maeno, H. V. Thuc, T. K. Anh, and M. Aikawa.** 1993. Differential sequestration of parasitized erythrocytes in the cerebrum and cerebellum in human cerebral malaria. Am. J. Trop. Med. Hyg. **48:**504–511.

96. **Senaldi, G. and P. F. Piguet.** 1998. Mortality and platelet depletion occur independently of fibrinogen consumption in murine models of tumor necrosis factor-mediated systemic inflammatory responses. Cytokine **10:**382–389.

97. **Shaffer, N., G. E. Grau, K. Hedberg, F. Davachi, B. Lyamba, A. W. Hightower, J. G. Breman, and P. Nguyen. Dinh.** 1991. Tumor necrosis factor and severe malaria. J. Infect. Dis. **163:**96–101.

98. **Sharma, Y. D.** 1997. Knob proteins in falciparum malaria. Indian J. Med. Res. **106:**53–62.

99. **Sherman, I. W., I. Crandall, and H. Smith.** 1992. Membrane proteins involved in the adherence of *Plasmodium falciparum*-infected erythrocytes to the endothelium. Biol. Cell **74:**161–178.

100. **Silamut, K., N. H. Phu, C. Whitty, G. D. H. Turner, K. Louwrier, N. T. H. Mai, J. A. Simpson, T. T. Hien, and N. J. White.** 1999. A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am. J. Pathol. **155:**395–410.

101. **Smith, C. D., A. E. Brown, S. Nakazawa, H. Fujioka, and M. Aikawa.** 1996. Multi-organ erythrocyte sequestration and ligand expression in rhesus monkeys infected with *Plasmodium coatneyi* malaria. Am. J. Trop. Med. Hyg. **55:**379–383.

102. **Smith, H., J. A. Nelson, C. G. Gahmberg, I. Crandall, and I. W. Sherman.** 1992. *Plasmodium falciparum*: cytoadherence of malaria-infected erythrocytes to human brain capillary and umbilical vein endothelial cells–a comparative study of adhesive ligands. Exp. Parasitol. **75:**269–280.

103. **Steinshamn, S., M. H. A. Bemelmans, L. J. H. Vantits, K. Bergh, W. A. Buurmann, and A. Waage.** 1996. TNF receptors in murine *Candida albicans* infection—evidence for an important role of TNF receptor p55 in antifungal defense. J. Immunol. **157:**2155–2159.

104. **Tacchini-Cottier, F., J. Lou, D. J. Roberts, A. M. Garcia, and G. E. Grau.** 1995. Detection of an LFA-1 epitope on the surface of erythrocytes infected with a strain of *P. falciparum*. Immunology **85:**205–213.

105. **Tatke, M., and G. Bazaz Malik.** 1989. Brain histomorphology in protein deprived rhesus monkeys with fatal malarial infection. Indian J. Med. Res. **89:**404–410.

106. **Tourneur, N., A. Scherf, M. Wahlgren, and J. Gysin.** 1992. The squirrel monkey as an experimental model for *Plasmodium falciparum* erythrocyte rosette formation. Am. J. Trop. Med. Hyg. **47:**633–642.

107. **Treutiger, C. J., A. Heddini, V. Fernandez, W. A. Muller, and M. Wahlgren.** 1997. PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes. Nat. Med. **3:**1405–1408.

108. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1986. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. J. Immunol. **136:** 2441–2444.

109. **Turner, G.** 1997. Cerebral malaria. Brain Pathol. **7:**569–582.

110. **Turner, G. D. H., H. Morrison, M. Jones, T. M. E. Davis, S. Looareesuwan, I. D. Buley, K. C. Gatter, C. I. Newbold, S. Pukritayakamee, B. Nagachinta, N. J. White, and A. R. Berendt.** 1994. An immunohistochemical study of the pathology of fatal malaria—evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am. J. Pathol. **145:**1057–1069.

111. **Udeinya, I. J.** 1990. In vitro and *ex vivo* models of sequestration in *Plasmodium-Falciparum* infection. Am. J. Trop. Med. Hyg. **43:**2–5.

112. **Udeinya, I. J., J. A. Schmidt, M. Aikawa, L. H. Miller, and I. Green.** 1981. Falciparum malaria-infected erythrocytes specifically bind to cultured endothelial cells. Science **213:**555–557.

113. **Udomsangpetch, R., M. Aikawa, K. Berzins, M. Wahlgren, and P. Perlmann.** 1989. Cytoadherence of knobless *Plasmodium falciparum*-infected erythrocytes and its inhibition by a human monoclonal antibody. Nature **338:**763–765.

114. **Udomsangpetch, R., P. H. Reinhardt, T. Schollaardt, J. F. Elliott, P. Kubes, and M. Ho.** 1997. Promiscuity of clinical *Plasmodium falciparum* isolates for multiple adhesion molecules under flow conditions. J. Immunol. **158:**4358–4364.

115. **van Hensbroek, M. B., A. Palmer, E. Onyiorah, G. Schneider, S. Jaffar, G. Dolan, H. Memming, J. Frenkel, G. Enwere, S. Bennett, D. Kwiatkowski, and B. Greenwood.** 1996. The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J. Infect. Dis. **174:**1091–1097.

116. **Vieira, L. Q., M. Goldschmidt, M. Nashleanas, K. Pfeffer, T. Mak, and P. Scott.** 1996. Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with *Leishmania major*, but control parasite replication. J. Immunol. **157:**827–835.

117. **Waki, S., S. Uehara, K. Kanbe, K. Ono, M. Suzuki, and H. Nariuchi.** 1992. The role of T-cells in pathogenesis and protective immunity to murine malaria. Immunology **75:**646–651.

118. **Warrell, D. A., M. E. Molyneux, and P. F. Beales.** 1990. Severe and complicated malaria. Trans. R. Soc. Trop. Med. Hyg. **84:**1–65.

119. **Wick, T. M., and V. Louis.** 1991. Cytoadherence of *Plasmodium-falciparum*-infected erythrocytes to human umbilical vein and human dermal microvascular endothelial cells under shear conditions. Am. J. Trop. Med. Hyg. **45:**578–586.

120. **Willimann, K., H. Matile, N. A. Weiss, and B. A. Imhof.** 1995. In vivo sequestration of *Plasmodium falciparum*-infected human erythrocytes: a severe combined immunodeficiency mouse model for cerebral malaria. J. Exp. Med. **182:**643–653.

121. **Xiao, L. H., C. F. Yang, K. Dorovinizia, N. N. Tandon, E. W. Ades, A. A. Lal, and V. Udhayakumar.** 1996. *Plasmodium falciparum*: involvement of additional receptors in the cytoadherence of infected erythrocytes to microvascular endothelial cells. Exp. Parasitol. **84:**42–55.

122. **Xiao, L. H., C. F. Yang, P. S. Patterson, V. Udhayakumar, and A. A. Lal.** 1996. Sulfated polyanions inhibit invasion of erythrocytes by plasmodial merozoites and cytoadherence of endothelial cells to parasitized erythrocytes. Infect. Immun. **64:**1373–1378.

123. **Yoeli, M., H. Most, B. Hargreaves, and L. Fajardo.** 1975. Cerebral malaria in a virulent rodent plasmodial infection. Trans. R. Soc. Trop. Med. Hyg. **69:**4–5.